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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS

(57) Abstract

Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells in situ and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration in vivo. Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.

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DESCRIPTION

Methods and Compositions for Stimulating Bone Cells

The present application is a continuation-in-part of U.S. Serial Number 08/316,650, filed September 30, 1994; which is a continuation-in-part of U.S. Serial Number 08/199,780, filed February 18, 1994; the entire text and figures of which disclosures are specifically incorporated herein by reference without disclaimer. The United States government has certain rights in the present invention pursuant to Grant HL-41926 from the National Institutes of Health.

15 1. Field of the Invention

The present invention relates generally to the field of bone cells and tissues. More particularly, certain embodiments concern the transfer of genetic material into bone and other embodiments concern type II collagen. In certain examples, the invention concerns the use of type II collagen and nucleic acids to stimulate bone growth, repair and regeneration. Methods, compositions, kits and devices are provided for transferring an osteotropic gene into bone progenitor cells, which is shown to stimulate progenitor cells and to promote increased bone formation in vivo.

2. Description of the Related Art

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Defects in the process of bone repair and regeneration are linked to the development of several human diseases and disorders, e.g., osteoporosis and osteogenesis imperfecta. Failure of the bone repair mechanism is, of course, also associated with significant complications in clinical orthopaedic practice, for example, fibrous non-union following bone fracture,

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implant interface failures and large allograft failures. The lives of many individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

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Naturally, any new technique to stimulate bone repair would be a valuable tool in treating bone fractures. A significant portion of fractured bones are still treated by casting, allowing natural mechanisms to effect wound repair. Although there have been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the wound repair mechanisms would represent significant progress in this area.

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A very significant patient population that would benefit from new therapies designed to promote fracture repair, or even prevent or lessen fractures, are those patients suffering from osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age.

An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. The cost of treating osteoporosis in the United States is currently estimated to be in the order of \$10 billion per year. Demographic trends, i.e., the gradually increasing age of the US population, suggest that these costs may increase 2-3 fold by the year 2020 if a safe and

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effective treatment is not found.

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The major focus of current therapies for osteoporosis is fracture prevention, not fracture repair. This is an important consideration, as it is known that significant morbidity and mortality are associated with prolonged bed rest in the elderly, especially those who have suffered hip fracture. New methods are clearly needed for stimulating fracture repair, thus restoring mobility in these patients before the complications arise.

Osteogenesis imperfecta (OI) refers to a group of inherited connective tissue diseases characterized by bone and soft connective tissue fragility (Byers and 15 Steiner, 1992; Prockop, 1990). Males and females are affected equally, and the overall incidence is currently estimated to be 1 in 5,000-14,000 live births. loss, dentinogenesis imperfecta, respiratory insufficiency, severe scoliosis and emphysema are just 20 some of the conditions that are associated with one or more types of OI. While accurate estimates of the health care costs are not available, the morbidity and mortality associated with OI certainly result from the extreme propensity to fracture (OI types I-IV) and the deformation of abnormal bone following fracture repair (OI types II-IV) (Bonadio and Goldstein, 1993). The most relevant issue with OI treatment is to develop new methods by which to improve fracture repair and thus to improve the quality of life of these patients.

The techniques of bone reconstruction, such as is used to reconstruct defects occurring as a result of trauma, cancer surgery or errors in development, would also be improved by new methods to promote bone repair. Reconstructive methods currently employed, such as using autologous bone grafts, or bone grafts with attached soft

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tissue and blood vessels, are associated with significant drawbacks of both cost and difficulty. For example, harvesting a useful amount of autologous bone is not easily achieved, and even autologous grafts often become infected or suffer from resorption.

The process of bone repair and regeneration resembles the process of wound healing in other tissues. A typical sequence of events includes; hemorrhage; clot formation; dissolution of the clot with concurrent 10 removal of damaged tissues; ingrowth of granulation tissue; formation of cartilage; capillary ingrowth and cartilage turnover; rapid bone formation (callus tissue); and, finally, remodeling of the callus into cortical and trabecular bone. Therefore, bone repair is a complex 15 process that involves many cell types and regulatory molecules. The diverse cell populations involved in fracture repair include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, 20 and osteoclasts.

Regulatory factors involved in bone repair are known to include systemic hormones, cytokines, growth factors, and other molecules that regulate growth and differentiation. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs), and have also been termed osteogenic bone inductive proteins or osteogenic proteins (OPs). Several BMP (or OP) genes have now been cloned, and the common designations are BMP-1 through BMP-8. New BMPs are in the process of discovery. Although the BMP terminology is widely used, it may prove to be the case that there is an OP

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counterpart term for every individual BMP (Alper, 1994).

BMPs 2-8 are generally thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). BMPs are related to, or part of, the transforming growth factor- β (TGF- β) superfamily, and both TGF- β 1 and TGF- β 2 also regulate osteoblast function (Seitz et al., 1992). Several BMP (or OP) nucleotide sequences and polypeptides have been described in U.S. Patents, e.g., 4,795,804; 4,877,864; 4,968,590; 5,108,753; including, specifically, BMP-1 disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; BMP-7 in 5,108,753 and 5,141,905; and OP-1, COP-5 and COP-7 in 5,011,691.

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Other growth factors or hormones that have been reported to have the capacity to stimulate new bone formation include acidic fibroblast growth factor (Jingushi et al., 1990); estrogen (Boden et al., 1989); macrophage colony stimulating factor (Horowitz et al., 1989); and calcium regulatory agents such as parathyroid hormone (PTH) (Raisz and Kream, 1983).

Several groups have investigated the possibility of using bone stimulating proteins and polypeptides, particularly recombinant BMPs, to influence bone repair in vivo. For example, recombinant BMP-2 has been employed to repair surgically created defects in the mandible of adult dogs (Toriumi et al., 1991), and high doses of this molecule have been shown to functionally repair segmental defects in rat femurs (Yasko et al., 1992). Chen and colleagues showed that a single

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application of 25-100 mg of recombinant TGF- β l adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). It has also been reported that an application of TGF- β l in a 34 methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991).

10 However, there are many drawbacks associated with these type of treatment protocols, not least the expensive and time-consuming purification of the recombinant proteins from their host cells. Also, polypeptides, once administered to an animal are more unstable than is generally desired for a therapeutic 15 agent, and they are susceptible to proteolytic attack. Furthermore, the administration of recombinant proteins can initiate various inhibitive or otherwise harmful immune responses. It is clear, therefore, that a new method capable of promoting bone repair and regeneration 20 in vivo would represent a significant scientific and medical advance with immediate benefits to a large number of patients. A method readily adaptable for use with a variety of matrices and bone-stimulatory genes would be particularly advantageous. 25

SUMMARY OF THE INVENTION

The present invention overcomes one or more of these and other drawbacks inherent in the prior art by providing novel methods, compositions and devices for use in transferring nucleic acids into bone cells and tissues, and for promoting bone repair and regeneration.

Certain embodiments of the invention rest, generally, with the inventors' surprising finding that nucleic acids can be effectively transferred to bone progenitor cells

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in vivo and that, in certain embodiments, the transfer of an osteotropic gene stimulates bone repair in an animal.

The invention, in general terms, thus concerns methods, compositions and devices for transferring a nucleic acid segment into bone progenitor cells or tissues. The methods of the invention generally comprise contacting bone progenitor cells with a composition comprising a nucleic acid segment in a manner effective to transfer the nucleic acid segment into the cells. The cells may be cultured cells or recombinant cells maintained in vitro, when all that is required is to add the nucleic acid composition to the cells, e.g., by adding it to the culture media.

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Alternatively, the progenitor cells may be located within a bone progenitor tissue site of an animal, when the nucleic acid composition would be applied to the site in order to effect, or promote, nucleic acid transfer into bone progenitor cells in vivo. In transferring nucleic acids into bone cells within an animal, a preferred method involves first adding the genetic material to a bone-compatible matrix and then using the resultant matrix to contact an appropriate tissue site within the animal. The "resultant" matrix may, in certain embodiments, be referred to as a matrix impregnated with genetic material, or it may take the form of a matrix-nucleic acid mixture, or even conjugate.

An extremely wide variety of genetic material can be transferred to bone progenitor cells or tissues using the compositions and methods of the invention. For example, the nucleic acid segment may be DNA (double or single-stranded) or RNA (e.g., mRNA, tRNA, rRNA); it may also be a "coding segment", i.e., one that encodes a protein or polypeptide, or it may be an antisense nucleic acid molecule, such as antisense RNA that may function to

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disrupt gene expression. The nucleic acid segments may thus be genomic sequences, including exons or introns alone or exons and introns, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone progenitor cell or tissue. Suitable nucleic acid segments may also be in virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids; functional inserts within the genomes of various recombinant viruses, including viruses with DNA genomes and retroviruses; and any form of nucleic acid segment, plasmid or virus associated with a liposome or a gold particle, the latter of which may be employed in connection with the gene gun technology.

The invention may be employed to promote expression 15 of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. expression could be increased expression of a gene that is normally expressed (i.e., "over-expression"), or it could be used to express a gene that is not normally 20 associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change 25 or alter the phenotype. Gene suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

30 1. Bone Progenitor Cells and Tissues

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In certain embodiments, this invention provides advantageous methods for using genes to stimulate bone progenitor cells. As used herein, the term "bone progenitor cells" refers to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. This includes

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various cells in different stages of differentiation, such as, for example, stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, osteoclasts, and the like. Bone progenitor cells also include cells that have been isolated and manipulated in vitro, e.g., subjected to stimulation with agents such as cytokines or growth factors or even genetically engineered cells. The particular type or types of bone progenitor cells that are stimulated using the methods and compositions of the invention are not important, so long as the cells are stimulated in such a way that they are activated and, in the context of in vivo embodiments, ultimately give rise to new bone tissue.

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15 The term "bone progenitor cell" is also used to particularly refer to those cells that are located within, are in contact with, or migrate towards (i.e., "home to"), bone progenitor tissue and which cells directly or indirectly stimulate the formation of mature bone. As such, the progenitor cells may be cells that 20 ultimately differentiate into mature bone cells themselves, i.e., cells that "directly" form new bone tissue. Cells that, upon stimulation, attract further progenitor cells or promote nearby cells to differentiate 25 into bone-forming cells (e.g., into osteoblasts, osteocytes and/or osteoclasts) are also considered to be progenitor cells in the context of this disclosure - as their stimulation "indirectly" leads to bone repair or regeneration. Cells affecting bone formation indirectly 30 may do so by the elaboration of various growth factors or cytokines, or by their physical interaction with other cell types. Although of scientific interest, the direct or indirect mechanisms by which progenitor cells

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stimulate bone or wound repair is not a consideration in practicing this invention.

Bone progenitor cells and bone progenitor tissues may be cells and tissues that, in their natural environment, arrive at an area of active bone growth, repair or regeneration (also referred to as a wound In terms of bone progenitor cells, these repair site). may also be cells that are attracted or recruited to such These may be cells that are present within an artificially-created osteotomy site in an animal model, such as those disclosed herein. Bone progenitor cells may also be isolated from animal or human tissues and maintained in an in vitro environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site), or indeed, from the bone Isolated cells may be stimulated using the methods and compositions disclosed herein and, if desired, be returned to an appropriate site in an animal where bone repair is to be stimulated. In such cases, the nucleic-acid containing cells would themselves be a form of therapeutic agent. Such ex vivo protocols are well known to those of skill in the art.

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In important embodiments of the invention, the bone progenitor cells and tissues will be those cells and tissues that arrive at the area of bone fracture or damage that one desires to treat. Accordingly, in treatment embodiments, there is no difficulty associated with the identification of suitable target progenitor cells to which the present therapeutic compositions should be applied. All that is required in such cases is to obtain an appropriate stimulatory composition, as disclosed herein, and contact the site of the bone fracture or defect with the composition. The nature of

this biological environment is such that the appropriate cells will become activated in the absence of any further targeting or cellular identification by the practitioner.

Certain methods of the invention involve, generally, 5 contacting bone progenitor cells with a composition comprising one or more osteotropic genes (with or without additional genes, proteins or other biomolecules) so as to promote expression of said gene in said cells. As outlined above, the cells may be contacted in vitro or in 10 This is achieved, in the most direct manner, by simply obtaining a functional osteotropic gene construct and applying the construct to the cells. The present inventors surprisingly found that there are no particular 15 molecular biological modifications that need to be performed in order to promote effective expression of the gene in progenitor cells. Contacting the cells with DNA, e.g., a linear DNA molecule, or DNA in the form of a plasmid or other recombinant vector, that contains the gene of interest under the control of a promoter, along 20 with the appropriate termination signals, is sufficient to result in uptake and expression of the DNA, with no further steps necessary.

In preferred embodiments, the process of contacting the progenitor cells with the osteotropic gene composition is conducted in vivo. Again, a direct consequence of this process is that the cells take up and express the gene and that they, without additional steps, function to stimulate bone tissue growth, repair or regeneration.

An assay of an osteoinductive gene may be conducted using the bone induction bioassay of Sampath and Reddi (1981; incorporated herein by reference). This is a rat bone formation assay that is routinely used to evaluate the osteogenic activity of bone inductive factors.

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However, for analyzing the effects of osteotropic genes on bone growth, one is generally directed to use the novel osteotomy model disclosed herein.

5 2. Osteotropic Genes

As used herein, the terms "osteotropic and osteogenic gene" are used to refer to a gene or DNA coding region that encodes a protein, polypeptide or 10 peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increases the rate of primary bone growth or healing (or even a gene that increases the rate of skeletal connective tissue growth or healing). The terms promoting, inducing and stimulating are used interchangeably throughout this text 15 to refer to direct or indirect processes that ultimately result in the formation of new bone tissue or in an increased rate of bone repair. Thus, an osteotropic gene is a gene that, when expressed, causes the phenotype of a 20 cell to change so that the cell either differentiates, stimulates other cells to differentiate, attracts boneforming cells, or otherwise functions in a manner that ultimately gives rise to new bone tissue.

25 In using the new osteotomy model of the invention, an osteotropic gene is characterized as a gene that is capable of stimulating proper bone growth in the osteotomy gap to any degree higher than that observed in control studies, e.g., parallel studies employing an 30 irrelevant marker gene such as β -galactosidase. This stimulation of "proper bone growth" includes both the type of tissue growth and the rate of bone formation. using the model with a 5 mm osteotomy gap, an osteotropic gene is generally characterized as a gene that is capable 35 of promoting or inducing new bone formation, rather than abnormal bone fracture repair, i.e., fibrous non-union. In using the 2 mm osteotomy gap, one may characterize

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osteotropic genes as genes that increase the rate of primary bone healing as compared to controls, and more preferably, genes capable of stimulating repair of the osteotomy defect in a time period of less than nine weeks.

In general terms, an osteotropic gene may also be characterized as a gene capable of stimulating the growth or regeneration of skeletal connective tissues such as, e.g., tendon, cartilage, and ligament. Thus, in certain embodiments, the methods and compositions of the invention may be employed to stimulate the growth or repair of both bone tissue itself and also of skeletal connective tissues.

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A variety of osteotropic genes are now known, all of which are suitable for use in connection with the present invention. Osteotropic genes and the proteins that they encode include, for example, systemic hormones, such as parathyroid hormone (PTH) and estrogen; many different growth factors and cytokines; chemotactic or adhesive peptides or polypeptides; molecules such as activin (U.S. Patent 5,208,219, incorporated herein by reference); specific bone morphogenetic proteins (BMPs); and even growth factor receptor genes.

Examples of suitable osteotropic growth factors include those of the transforming growth factor (TGF) gene family, including TGFs 1-3, and particularly TGF- β 1, TGF- β 2 and TGF- β 3, (U.S. Patents 4,886,747 and 4,742,003, incorporated herein by reference), with TGF- α (U.S. Patent 5,168,051, incorporated herein by reference) also being of possible use; and also fibroblast growth factors (FGF), previously referred to as acidic and basic FGF and now referred to as FGF1-9; granulocyte/macrophage colony stimulating factor (GMCSF); epidermal growth factor (EGF); platelet derived growth factor (PDGF); insulin-

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like growth factors (IGF), including IGF-I and IGF-II; and leukemia inhibitory factor (LIF), also known as HILDA and DIA. Any of the above or other related genes, or DNA segments encoding the active portions of such proteins, may be used in the novel methods and compositions of the invention.

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Certain preferred osteotropic genes and DNA segments are those of the TGF superfamily, such as TGF- β 1, TGF- β 2, TGF- β 3 and members of the BMP family of genes. For example, several BMP genes have been cloned that are ideal candidates for use in the nucleic acid transfer or delivery protocols of the invention. Suitable BMP genes are those designated BMP-2 through BMP-12. BMP-1 is not considered to be particularly useful at this stage. 15

There is considerable variation in the terminology currently employed in the literature in referring to these genes and polypeptides. It will be understood by 20 those of skill in the art that all BMP genes that encode an active osteogenic protein are considered for use in this invention, regardless of the differing terminology that may be employed. For example, BMP-3 is also called osteogenin and BMP-7 is also called OP-1 (osteogenic protein-1). It is likely that the family of factors 25 termed OP(s) is as large as that termed BMP(s), and that these terms, in fact, describe the same set of molecules (Alper, 1994).

30 The DNA sequences for several BMP (or OP) genes have been described both in scientific articles and in U.S. Patents such as 4,877,864; 4,968,590; 5,108,753. Specifically, BMP-1 sequences are disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) 35 in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6

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in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference). The article by Wozney et al., (1988; incorporated herein by reference) is considered to be particularly useful for describing BMP molecular clones and their activities. DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691.

All of the above issued U.S. Patents are 10 incorporated herein by reference and are intended to be used in order to supplement the present teachings regarding the preparation of BMP and OP genes and DNA segments that express osteotropic polypeptides. disclosed in the above patents, and known to those of 15 skill in the art, the original source of a recombinant gene or DNA segment to be used in a therapeutic regimen need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant PTH, TGF or BMP gene may be employed to 20 promote bone repair or regeneration in a human subject or an animal, e.g., a horse. Particularly preferred genes are those from human, murine and bovine sources, in that such genes and DNA segments are readily available, with the human or murine forms of the gene being most 25 preferred for use in human treatment regimens. Recombinant proteins and polypeptides encoded by isolated DNA segments and genes are often referred to with the prefix "r" for recombinant and "rh" for recombinant human. As such, DNA segments encoding rBMPs, such as rhBMP-2 or rhBMP-4, are contemplated to be particularly 30 useful in connection with this invention.

The definition of a *BMP gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions (see, e.g., Maniatis et al., 1982), to DNA sequences presently known to include BMP gene sequences.

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To prepare an osteotropic gene segment or cDNA one may follow the teachings disclosed herein a lalso the teachings of any of patents or scientific documents specifically referenced herein. Various nucleotide sequences encoding active BMPs are disclosed in U.S. 5 Patents 5,166,058, 5,013,649, 5,116,738, 5,106,748, 5,187,076, 5,108,753 and 5,011,691, each incorporated herein by reference. By way of example only, U.S. Patent 5,166,058, teaches that hBMP-2 is encoded by a nucleotide 10 sequence from nucleotide #356 to nucleotide #1543 of the sequence shown in Table II of the patent. One may thus obtain a hBMP-2 DNA segment using molecular biological techniques, such as polymerase chain reaction (PCR™) or screening a cDNA or genomic library, using primers or 15 probes with sequences based on the above nucleotide sequence. The practice of such techniques is a routine matter for those of skill in the art, as taught in various scientific articles, such as Sambrook et al., (1989), incorporated herein by reference. Certain 20 documents further particularly describe suitable mammalian expression vectors, e.g., U.S. Patent 5,168,050, incorporated herein by reference.

Osteotropic genes and DNA segments that are

particularly preferred for use in certain aspects of the present compositions and methods are the TGF, PTH and BMP genes. TGF genes are described in U.S. Patents 5,168,051; 4,886,747 and 4,742,003, each incorporated herein by reference. TGFα may not be as widely applicable as TGFβ, but is proposed for use particularly in applications involving skeletal soft tissues. The PTH gene, or a DNA segment encoding the active fragment thereof, such as a DNA segment encoding a polypeptide that includes the amino acids 1-34 (hPTH1-34; Hendy et al., 1981; incorporated herein by reference) is another

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preferred gene; as are the BMP genes termed BMP-4 and BMP-2, such as the gene or cDNA encoding the murine BMP-4 disclosed herein.

5 It is also contemplated that one may clone further genes or cDNAs that encode an osteotropic protein or polypeptide. The techniques for cloning DNA molecules, i.e., obtaining a specific coding sequence from a DNA library that is distinct from other portions of DNA, are 10 well known in the art. This can be achieved by, for example, screening an appropriate DNA library, as. disclosed in Example XV herein, which relates to the cloning of a wound healing gene. The screening procedure may be based on the hybridization of oligonucleotide 15 probes, designed from a consideration of portions of the amino acid sequence of known DNA sequences encoding related osteogenic proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, in Sambrook et al., (1989), 20 incorporated herein by reference.

Osteotropic genes with sequences that vary from those described in the literature are also encompassed by the invention, so long as the altered or modified gene still encodes a protein that functions to stimulate bone progenitor cells in any direct or indirect manner. These sequences include those caused by point mutations, those due to the degeneracies of the genetic code or naturally occurring allelic variants, and further modifications that have been introduced by genetic engineering, i.e., by the hand of man.

Techniques for introducing changes in nucleotide
sequences that are designed to alter the functional
properties of the encoded proteins or polypeptides are
well known in the art, e.g., U.S. Patent 4,518,584,

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incorporated herein by reference, which techniques are also described in further detail herein. Such modifications include the deletion, insertion or substitution of bases, and thus, changes in the amino acid sequence. Changes may be made to increase the osteogenic activity of a protein, to increase its biological stability or half-life, to change its glycosylation pattern, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

It will, of course, be understood that one or more than one osteotropic gene may be used in the methods and compositions of the invention. The nucleic acid delivery 15 methods may thus entail the administration of one, two, three, or more, osteotropic genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting a 20 significant adverse cytotoxic effect. The particular combination of genes may be two or more distinct BMP genes; or it may be such that a growth factor gene is combined with a hormone gene, e.g., a BMP gene and a PTH gene; a hormone or growth factor gene may even be 25 combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same of different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell stimulation and bone growth,

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any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic segment or gene could be administered in combination with further agents, such as, e.g., proteins or polypeptides or various pharmaceutically active So long as genetic material forms part of the composition, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse 15 effect upon contact with the target cells or tissues. The nucleic acids may thus be delivered along with various other agents, for example, in certain embodiments one may wish to administer an angiogenic factor, and/or 20 an inhibitor of bone resorption, as disclosed in U.S. Patents 5,270,300 and 5,118,667, respectively, each incorporated herein by reference.

3. Gene Constructs and DNA Segments

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As used herein, the terms "gene" and "DNA segment" are both used to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a gene or DNA segment encoding an osteotropic gene refers to a DNA segment that contains sequences encoding an osteotropic protein, but is isolated away from, or purified free from, total genomic DNA of the species from which the DNA is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like. - 20 -

The term "gene" is used for simplicity to refer to a functional protein or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, an osteotropic gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturallyoccurring coding DNA, such as large chromosomal fragments 10 or other functional genes or cDNA coding regions. course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions, such as sequences encoding leader peptides or targeting 15 sequences, later added to the segment by the hand of man.

This invention provides novel ways in which to utilize various known osteotropic DNA segments and recombinant vectors. As described above, many such vectors are readily available, one particular detailed 20 example of a suitable vector for expression in mammalian cells is that described in U.S. Patent 5,168,050, incorporated herein by reference. However, there is no requirement that a highly purified vector be used, so long as the coding segment employed encodes a osteotropic 25 protein and does not include any coding or regulatory sequences that would have a significant adverse effect on bone progenitor cells. Therefore, it will also be understood that useful nucleic acid sequences may include 30 additional residues, such as additional non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

After identifying an appropriate osteotropic gene or DNA molecule, it may be inserted into any one of the many vectors currently known in the art, so that it will

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direct the expression and production of the osteotropic protein when incorporated into a bone progenitor cell. In a recombinant expression vector, the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with an osteotropic gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR[™] technology, in connection with the compositions disclosed herein.

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In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or 15 heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an osteotropic gene in its natural environment. Such promoters may include 20 those normally associated with other osteotropic genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in bone 25 progenitor cells.

The use of recombinant promoters to achieve protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., (1989). The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level or regulated expression of the introduced DNA segment. The currently preferred promoters are those such as CMV, RSV LTR, the SV40 promoter alone, and the SV40 promoter in combination with various enhancer elements.

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Osteotropic genes and DNA segments may also be in the form of a DNA insert which is located within the genome of a recombinant virus, such as, for example a recombinant adenovirus, adeno-associated virus (AAV) or retrovirus. In such embodiments, to place the gene in contact with a bone progenitor cell, one would prepare the recombinant viral particles, the genome of which includes the osteotropic gene insert, and simply contact the progenitor cells or tissues with the virus, whereby the virus infects the cells and transfers the genetic material.

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In certain preferred embodiments, one would impregnate a matrix or implant material with virus by soaking the material in recombinant virus stock solution, e.g., for 1-2 hours, and then contact the bone progenitor cells or tissues with the resultant, impregnated matrix. Cells then penetrate, or grow into, the matrix, thereby contacting the virus and allowing viral infection which leads to the cells taking up the desired gene or cDNA and expressing the encoded protein.

In other preferred embodiments, one would form a matrix-nucleic acid admixture, whether using naked DNA, a 25 plasmid or a viral vector, and contact the bone progenitor cells or tissues with the resultant admixed matrix. The matrix may then deliver the nucleic acid into the cells following disassociation at the cell surface, or in the immediate cellular environment. 30 Equally, the matrix admixture itself, especially a particle- or fiber-DNA admixture, may be taken up by cells to provide subsequent intracellular release of the genetic material. The matrix may then be extruded from the cell, catabolized by the cell, or even stored within the cell. The molecular mechanism by which a bone-35 compatible matrix achieves transfer of DNA to a cell is immaterial to the practice of the present invention.

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4. Bone-Compatible Matrices

In certain preferred embodiments, the methods of the invention involved preparing a composition in which the osteotropic gene, genes, DNA segments, or cells already incorporating such genes or segments, are associated with, impregnated within, or even conjugated to, a bone-compatible matrix, to form a "matrix-gene composition" and the matrix-gene composition is then placed in contact with the bone progenitor cells or tissue. The matrix may become impregnated with a gene DNA segment simply by soaking the matrix in a solution containing the DNA, such as a plasmid solution, for a brief period of time of anywhere from about 5 minutes or so, up to and including about two weeks.

Matrix-gene compositions are all those in which genetic material is adsorbed, absorbed, impregnated, conjugated to, or otherwise generally maintained in contact with the matrix. "Maintained in contact with the matrix" means that an effective amount of the nucleic acid composition should remain functionally associated with the matrix until its transfer to the bone progenitor cell or its release in the bone tissue site.

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The type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless, so long as it is a "bone-compatible matrix". This means that the matrix has all the features commonly associated with being "biocompatible", in that it is in a form that does not produce a significant adverse, allergic or other untoward reaction when administered to an animal, and that it is also suitable for placing in contact with bone tissue. A "significant" adverse effect is one that exceeds the normally accepted side-effects associated with any given therapy.

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"Bone-compatible", as used herein, means that the matrix (and gene) does not produce a significant adverse or untoward reaction when placed in contact with bone. In certain embodiments, when electing to use a particular bone compatible matrix, one may, optionally, take various other factors into consideration, for example, the capacity of the matrix to provide a structure for the developing bone, its capacity to be resorbed into the body after the bone has been repaired, and such like. However, these properties are not required to practice the invention and are merely exemplary of the factors that may be considered.

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In other embodiments, one may also consider the 15 likelihood that the matrix will be transported into the cell, e.g., by active or passive membrane transport. Where such transport and subsequent nucleic acid release is contemplated, other properties of the matrix and gene may be assessed in optimizing the matrix-gene formulation. For example, adenovirus vectors may provide 20 for advantageous DNA release in such embodiments. Matrices that are readily metabolized in the cytoplasm would also likely be preferred in such embodiments. Matrices that are later released from the cell, and preferably, also removed from the surrounding tissue 25 area, would be another preferred form of matrix for use in such embodiments.

The choice of matrix material will differ according

to the particular circumstances and the site of the bone
that is to be treated. Matrices such as those described
in U.S. Patent 5,270,300 (incorporated herein by
reference) may be employed. Physical and chemical
characteristics, such as, e.g., biocompatibility,

biodegradability, strength, rigidity, interface
properties, and even cosmetic appearance, may be
considered in choosing a matrix, as is well known to

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those of skill in the art. Appropriate matrices will deliver the gene composition and, in certain circumstances, may be incorporated into a cell, or may provide a surface for new bone growth, i.e., they may act as an in situ scaffolding through which progenitor cells may migrate.

A particularly important aspect of the present invention is its use in connection with orthopaedic implants and interfaces and artificial joints, including 10 implants themselves and functional parts of an implant, such as, e.g., surgical screws, pins, and the like. preferred embodiments, it is contemplated that the metal surface or surfaces of an implant or a portion thereof, such as a titanium surface, will be coated with a 15 material that has an affinity for nucleic acids, most preferably, with hydroxyl apatite, and then the coatedmetal will be further coated with the gene or nucleic acid that one wishes to transfer. The available chemical 20 groups of the absorptive material, such as hydroxyl apatite, may be readily manipulated to control its affinity for nucleic acids, as is known to those of skill in the art.

In certain embodiments, non-biodegradable matrices may be employed, such as sintered hydroxylapatite, aluminates, other bioceramic materials and metal materials, particularly titanium. A suitable ceramic delivery system is that described in U.S. Patent 4,596,574, incorporated herein by reference. Polymeric matrices may also be employed, including acrylic ester polymers, lactic acid polymers, and polylactic polyglycolic acid (PLGA) block copolymers, have been disclosed (U.S. Patent 4,526,909, U.S. Patent 4,563,489, Simons et al., 1992, and Langer and Folkman, 1976, respectively, each incorporated herein by reference).

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In certain embodiments, it is contemplated that a biodegradable matrix will likely be most useful. A biodegradable matrix is generally defined as one that is capable of being resorbed into the body. Potential biodegradable matrices for use in connection with the compositions, devices and methods of this invention include, for example, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxylapatite, PLGA block copolymers, polyanhydrides, matrices of purified proteins, and semi-purified extracellular matrix compositions.

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One preferred group of matrices are collagenous matrices, including those obtained from tendon or dermal collagen, e.g., type I collagen, which is generally prepared from dermis; those obtained from cartilage, such as type II collagen; and various other types of collagen. Collagens may be obtained from a variety of commercial sources, e.g., Sigma that supplies type II collagen obtained from bovine trachea; and Collagen Corporation. Collagen matrices may also be prepared as described in U.S. Patents 4,394,370 and 4,975,527, each incorporated herein by reference.

The various collagenous materials may also be in the form of mineralized collagen. One preferred mineralized collagenous material is that termed UltraFiber^m, obtainable from Norian Corp. (Mountain View, CA). U.S. Patent 5,231,169, incorporated herein by reference, describes the preparation of mineralized collagen through the formation of calcium phosphate mineral under mild agitation in situ in the presence of dispersed collagen fibrils. Such a formulation may be employed in the

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context of delivering a nucleic acid segment to a bone tissue site.

Certain other preferred collagenous materials are 5 those based upon type II collagen. Type II collagen preparations have been discovered to have the surprising and advantageous property of, absent any osteotropic gene, being capable of stimulating bone progenitor cells. Prior to the present invention, it was thought that 10 type II collagen only had a structural role in the cartilage extracellular matrix and the present finding that type II collagen is actually an osteoconductive/osteoinductive material is unexpected. The present invention thus contemplates the use of a 15 variety of type II collagen preparations as gene transfer matrices or bone cell stimulants, either with or without DNA segments, including native type II collagen, as prepared from cartilage, and recombinant type II collagen.

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PLGA block copolymers may also be employed as gene transfer matrices. Such polymers have been shown to readily incorporate DNA, are commercially available, non-toxic, and hydrolyze at defined rates, (i.e. they facilitate the sustained release of pharmaceutical agents). PLGA block copolymers have two particular advantageous properties in that, first, they exhibit reversible thermal gelation, and second, may be combined with other agents that allow for radiographic visualization.

5. Nucleic Acid Transfer Embodiments

Once a suitable matrix-gene composition has been
prepared or obtained, all that is required to deliver the osteotropic gene to bone progenitor cells within an animal is to place the matrix-gene composition in contact

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with the site in the body in which one wishes to promote bone growth. This may be achieved by physically positioning the matrix-gene composition in contact with the body site, or by injecting a syringeable form of the matrix-gene composition into the appropriate area.

The matrix-gene composition may be applied to a simple bone fracture site that one wishes to repair, an area of weak bone, such as in a patient with osteoporosis, or a bone cavity site that one wishes to fill with new bone tissue. Bone cavities may arise as a result of an inherited disorder, birth defect, or may result following dental or periodontal surgery or after the removal of an osteosarcoma.

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The use of PLGA and like compounds as matrices allows the matrix-DNA composition to be syringeable, which is achieved by, generally, admixing the matrix-gene composition with a pluronic agent. The resultant matrix-gene-pluronic may be stored within a thermal-jacket syringe, maintained at a temperature of about 4°C, immediately prior to administration to the body. In this temperature and environment, the composition will be a liquid. Following insertion into the body, the composition will equilibrate towards body temperature, and in so-doing will form a gelatinous matrix.

The above phenomenon is termed "reversible thermal gelation", and this allows for a controlled rate of gelation to be achieved. The manner of using pluronic agents in this context will be known to those of skill in the art in light of the present disclosure. Matrix-gene-pluronic compositions may also be admixed, or generally associated with, an imaging agent so that the present gene transfer technology may be used in imaging modalities. In these cases, the attending physician or veterinarian will be able to monitor the delivery and

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positioning of the matrix-gene composition. Many safe and effective imaging agents, such as the radiographic compound calcium phosphate, are available that may be used in conjunction with fluoroscopy, or even with tomography, to image the body or tissue site while the composition is being delivered.

Where an image of the tissue site is to be provided, one will desire to use a detectable imaging agent, such as a radiographic agent, or even a paramagnetic or radioactive agent. Many radiographic diagnostic agents are known in the art to be useful for imaging purposes, including e.g., calcium phosphate.

In the case of paramagnetic ions, examples include chromium (III), manganese (II), iron (III), iron (III), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being generally preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to, lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Although not generally preferred, radioactive isotopes are not excluded and may be used for imaging purposes if desired. Suitable ions include iodine¹³¹, iodine¹²³, technicium^{99m}, indium¹¹¹, rhenium¹⁸⁶, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ and astatine²¹¹.

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The amount of gene construct that is applied to the matrix and the amount of matrix-gene material that is applied to the bone tissue will be determined by the attending physician or veterinarian considering various biological and medical factors. For example, one would wish to consider the particular osteotropic gene and matrix, the amount of bone weight desired to be formed,

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the site of bone damage, the condition of the damaged bone, the patient's or animal's age, sex, and diet, the severity of any infection, the time of administration and any further clinical factors that may affect bone growth, such as serum levels of various factors and hormones. The suitable dosage regimen will therefore be readily determinable by one of skill in the art in light of the present disclosure, bearing in mind the individual circumstances.

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In treating humans and animals, progress may be monitored by periodic assessment of bone growth and/or repair, e.g., using X-rays. The therapeutic methods and compositions of the invention are contemplated for use in both medical and veterinary applications, due to the lack of species specificity in bone inductive factors. In particular, it is contemplated that domestic, farm and zoological animals, as well as thoroughbred horses, would be treatable using the nucleic acid transfer protocols disclosed herein.

The present methods and compositions may also have prophylactic uses in closed and open fracture reduction and also in the improved fixation of artificial joints. The invention is applicable to stimulating bone repair in congenital, trauma-induced, or oncologic resection-induced craniofacial defects, and also is useful in the treatment of periodontal disease and other tooth repair processes and even in cosmetic plastic surgery. The matrix-gene compositions and devices of this invention may also be used in wound healing and related tissue repair, including, but not limited to healing of burns, incisions and ulcers.

The present invention also encompasses DNA-based compositions for use in cellular transfer to treat bone defects and disorders. The compositions of the invention

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generally comprise an osteotropic gene in association with a bone-compatible matrix, such as type II collagen, wherein the composition is capable of stimulating bone growth, repair or regeneration upon administration to, or implantation within, a bone progenitor tissue site of an animal. The osteotropic gene or genes may be any of those described above, with $TGF-\alpha$ (for soft skeletal tissues), $TGF-\beta1$, $TGF-\beta2$, $TGF-\beta3$, PTH, BMP-2 and BMP-4 genes being generally preferred. Likewise, irrespective of the choice of gene, the bone-compatible matrix may be any of those described above, with biodegradable matrices such as collagen and, more particularly, type II collagen, being preferred.

15 In still further embodiments, the present invention concerns osteotropic devices, which devices may be generally considered as molded or designed matrix-gene compositions. The devices of the invention naturally comprise a bone-compatible matrix in which an osteotropic gene is associated with the matrix. The combination of 20 genes and matrix components is such that the device is capable of stimulating bone growth or healing when implanted in an animal. The devices may be of virtually any size or shape, so that their dimensions are adapted to fit a bone fracture or bone cavity site in the animal that is to be treated, allowing the fracture join and/or bone regrowth to be more uniform. Other particularly contemplated devices are those that are designed to act as an artificial joint. Titanium devices and hydroxylapatite-coated titanium devices will be preferred 30 in certain embodiments. Parts of devices in combination with an osteotropic nucleic acid segment, such as a DNAcoated screw for an artificial joint, and the like, also fall within the scope of the invention.

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Therapeutic kits comprising, in suitable container means, a bone compatible matrix, such as type II collagen

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or a PLGA block polymer, and an osteotropic gene form another aspect of the invention. Such kits will generally contain a pharmaceutically acceptable formulation of the matrix and a pharmaceutically acceptable formulation of an osteotropic gene, such as PTH, BMP, TGF- β , FGF, GMCSF, EGF, PDGF, IGF or a LIF gene. Currently preferred genes include PTH, TGF- β 1, TGF- β 2, TGF- β 3, and BMP-4 genes.

The kits may comprise a single container means that 10 contains both the biocompatible matrix and the osteotropic gene. The container means may, if desired, contain a pharmaceutically acceptable sterile syringeable matrix, having associated with it, the osteotropic gene composition and, optionally, a detectable label or 15 imaging agent. The syringeable matrix-DNA formulation may be in the form of a gelatinous composition, e.g., a type II collagen-DNA composition, or may even be in a more fluid form that nonetheless forms a gel-like composition upon administration to the body. 20 In these cases, the container means may itself be a syringe, pipette, or other such like apparatus, from which the matrix-DNA material may be applied to a bone tissue site or wound area. However, the single container means may contain a dry, or lyophilized, mixture of a matrix and 25 osteotropic gene composition, which may or may not require pre-wetting before use.

Alternatively, the kits of the invention may

comprise distinct container means for each component. In
such cases, one container would contain the osteotropic
gene, either as a sterile DNA solution or in a
lyophilized form, and the other container would include
the matrix, which may or may not itself be pre-wetted

with a sterile solution, or be in a gelatinous, liquid or
other syringeable form.

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The kits may also comprise a second or third container means for containing a sterile, pharmaceutically acceptable buffer, diluent or solvent. Such a solution may be required to formulate either the DNA component, the matrix component, both components separately, or a pre-mixed combination of the components, into a more suitable form for application to the body. e.g., a more gelatinous form. It should be noted. however, that all components of a kit could be supplied in a dry form (lyophilized), which would allow for "wetting" upon contact with body fluids. Thus, the presence of any type of pharmaceutically acceptable buffer or solvent is not a requirement for the kits of the invention. The kits may also comprise a second or third container means for containing a pharmaceutically acceptable detectable imaging agent or composition.

The container means will generally be a container such as a vial, test tube, flask, bottle, syringe or other container means, into which the components of the kit may placed. The matrix and gene components may also be aliquoted into smaller containers, should this be desired. The kits of the present invention may also include a means for containing the individual containers in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials or syringes are retained.

Irrespective of the number of containers, the kits
of the invention may also comprise, or be packaged with,
an instrument for assisting with the placement of the
ultimate matrix-gene composition within the body of an
animal. Such an instrument may be a syringe, pipette,
forceps, or any such medically approved delivery vehicle.

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6. Type II Collagen as an Osteoconductive/inductive Material

The present invention also provides methods for stimulating bone progenitor cells, as may be applied, in certain circumstances, to promote new bone formation, or to stimulate wound-healing. As such, the bone progenitor cells that are the targets of the invention may also be termed "wound healing bone progenitor cells". Although the function of wound healing itself may not always be required to practice all aspects of the invention, and although a mechanistic understanding is not necessary to practice the invention, it is generally thought that the wound healing process does operate during execution of the invention.

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To stimulate a bone progenitor cell in accordance with these aspects of the invention, generally one would contact a bone progenitor cell with a composition 20 comprising a biologically effective amount of type II collagen. Although preparations of crushed bone and mineralized collagen have been shown to be osteoconductive, this property has not previously been ascribed to type II collagen. The present inventors have 25 found that type II collagen alone is surprisingly effective at promoting new bone formation, it being able to bridge a 5 mm osteotomy gap in only eight weeks in all animals tested (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, and FIG. . 30 8C).

The forms of type II collagen that may be employed in this invention are virtually limitless. For example, type II collagen may be purified from hyaline cartilage of bovine trachea, or as isolated from diarthrodial joints or growth plates. Purified type II collagen is commercially available and may be purchased from, e.g., Sigma Chemical Company, St. Louis, MO. Any form of

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recombinant type II collagen may also be employed, as may be obtained from a type II collagen-expressing recombinant host cell, including bacterial, yeast, mammalian, and insect cells. One particular example of a recombinant type II collagen expression system is a yeast cell that includes an expression vector that encodes type II collagen, as disclosed herein in Example VI.

The type II collagen used in the invention may, if

desired, be supplemented with additional minerals, such
as calcium, e.g., in the form of calcium phosphate. Both
native and recombinant type II collagen may be
supplemented by admixing, adsorbing, or otherwise
associating with, additional minerals in this manner.

Such type II collagen preparations are clearly
distinguishable from the types of "mineralized collagen"
previously described, e.g., in U.S. Patent 5,231,169 that
describes the preparation of mineralized total collagen
fibrils.

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An object of this aspect of the invention is to provide a source of osteoconductive matrix material, that may be reproducibly prepared in a straightforward and cost-effective manner, and that may be employed, with or without an osteotropic gene segment, to stimulate bone progenitor cells. Recombinant type II collagen was surprisingly found to satisfy these criteria. Although clearly not required for effective results, the combination of native or recombinant type II collagen with mineral supplements, such as calcium, is encompassed by this invention.

A biologically effective amount of type II collagen is an amount of type II collagen that functions to stimulate a bone progenitor cell, as described herein. By way of example, one measure of a biologically effective amount is an amount effective to stimulate bone progenitor cells to the extent that new bone formation is evident. In this regard, the inventors have shown that 10 mg of lyophilized collagen functions effectively to close a 5 mm osteotomy gap in three weeks. This information may be used by those of skill in the art to optimize the amount of type II collagen needed for any given situation.

Depending on the individual case, the artisan would, in light of this disclosure, readily be able to calculate 10 an appropriate amount, or dose, of type II collagen for stimulating bone cells and promoting bone growth. terms of small animals or human subjects, suitable effective amounts of collagen include between about 1 mg and about 500 mg, and preferably, between about 1 mg and 15 about 100 mg, of lyophilized type II collagen per bone tissue site. Of course, it is likely that there will be variations due to, e.g., individual responses, particular tissue conditions, and the speed with which bone formation is required. While 10 mg were demonstrated to 20 be useful in the illustrative example, the inventors contemplate that 1, 5, 10, 15, 20, 30, 40, 50, 75, 100, 125, 150, 200, 300 mg, and the like, may be usefully employed for human patients and small animals. course, any values within these contemplated ranges may 25 be useful in any particular case.

Naturally, one of the main variables to be accounted for is the amount of new bone that needs to be generated in a particular area or bone cavity. This can be largely a function of the size of the animal to be treated, e.g., a cat or a horse. Therefore, there is currently no upper limit on the amount of type II collagen, or indeed on the amount of any matrix-gene composition, that can be employed in the methods of the invention, given careful supervision by the practitioner.

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In contacting or applying type II collagen, with or without a DNA segment, to bone progenitor cells located within a bone progenitor tissue site of an animal, bone tissue growth will be stimulated. Thus, bone cavity sites and bone fractures may be filled and repaired.

The use of type II collagen in combination with a nucleic acid segment that encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells is preferred, as described above. Nucleic acid segments that comprise an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or a chemotactic factor gene are preferred, with PTH, TGF- β and BMP genes being most preferred. The genes function subsequent to their transfer into, and expression in, bone progenitor cells of the treated animal, thus promoting bone growth.

Although type II collagen alone is effective, its

combined use with an osteotropic gene segment may prove
to give synergistic and particularly advantageous
effects. Type II collagen, whether native or
recombinant, may thus also be formulated into a
therapeutic kit with an osteotropic gene segment, in

accordance with those kits described herein above. This
includes the use of single or multiple container means,
and combination with any medically approved delivery
vehicle, including, but not limited to, syringes,
pipettes, forceps, additional diluents, and the like.

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BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification
and are included to further demonstrate certain aspects
of the present invention. The invention may be better
understood by reference to one or more of these drawings

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in combination with the detailed description of specific embodiments presented herein.

FIG. 1. A model of DNA therapy for bone repair.

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- FIG. 2A. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells in vivo. Shown is the method of creating osteotomy and placing gene-activated matrix in situ.
- FIG. 2B. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells in vivo. Shown is the method of fracturing repair cells where blood vessels grow into the gene-activated matrix (FIG. 2A).
- FIG. 2C. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells in vivo. Shown are fractured cells taking up DNA as an episomal element, i.e. direct gene transfer in vivo.
- FIG. 2D. A schematic model of the cellular and
 molecular basis of the direct DNA transfer mechanism into
 osteogenic cells in vivo. Shown are fractured repair
 synthesizing and secreting recombinant proteins encoded
 by the episomal DNA.
- FIG. 2E. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells in vivo. Shown is the resulting new bone formation.
- FIG. 3A. Achilles' tendon gene transfer is shown as a time course overview at 3 weeks post-surgery.

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FIG. 3B. Achilles' tendon gene transfer is shown as a time course overview at 9 weeks post-surgery.

- FIG. 3C. Achilles' tendon gene transfer is shown as a time course overview at 12 weeks post-surgery.
- FIG. 3D. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant impregnated with expression plasmid DNA. Note the positive cytoplasmic staining of fibroblastic cells 9 weeks post-surgery.
- FIG. 3E. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant alone, without DNA. Note the relative absence of cytoplasmic staining.
- FIG. 4. Monitoring of cruciate ligament gene transfer using a substrate utilization assay. Three weeks following the implantation of SIS soaked in a solution of the pSV40β-gal expression plasmid, tendon tissue was harvested, briefly fixed in 0.5% glutaraldehyde, and then incubated with X-gal according to published methods. Tissues were then embedded in paraffin and sections were cut and stained with H and E. Note the positive (arrows) staining in the cytoplasm of granulation tissues fibroblasts.
 - FIG. 5A. Direct DNA transfer into regenerating bone: β -gal activity. The figure compares β -galactosidase activity in homogenates of osteotomy gap tissue from two Sprague-Dawley rats. In animal #1, the UltraFiber[™] implant material was soaked in a solution of pSV40 β -gal DNA, Promega) encoding bacterial β -galactosidase. In animal #2, the implant material was

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soaked in a pure solution of pGL2-Promoter Vector DNA (Promega) encoding insect luciferase. Enzyme activity was determined using substrate assay kits (β -galactosidase and Luciferase Assay Systems, Promega). Note that significant β -galactosidase activity was found only in the homogenate prepared from animal #1.

pig. 5B. Direct DNA transfer into regenerating bone: luciferase activity. The figure compares

luciferase activity in aliquots of the homogenates described in FIG. 5A. Luciferase activity was determined using the commercial reagents and protocols (Promega) described in FIG. 5A. Note that significant luciferase activity is found only in the homogenate prepared from animal #2.

- FIG. 6A. Osteotomy gene transfer monitored by PTH studies. In this study an expression plasmid coding for a functional 34 amino acid peptide fragment of human parathyroid hormone (PTH1-34) was transferred and expressed in vivo using the GAM technology. The progress of new bone formation in the gap was monitored radiographically for three weeks and the animals were sacrificed. Shown is a radiograph of the osteotomy gap of the control animal that received an antisense hPTH1-34 GAM construct. There was no evidence of radiodense tissue in the gap.
- FIG. 6B. Osteotomy gene transfer (FIG. 6A)

 monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.
- FIG. 6C. Osteotomy gene transfer (FIG. 6A)
 monitored by PTH studies. Shown is a radiograph of the
 osteotomy gap that received the sense PTH1-34 GAM

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construct. Note the presence of radiodense tissue in the gap (arrow).

FIG. 6D. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of trabecular bone plates that extend into the gap from the surgical margin.

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FIG. 7A. Osteotomy gene transfer BMP-4 studies. Shown is immunohistochemical evidence of BMP-4 transgene expression by granulation tissue fibroblasts near the center of an osteotomy gap three weeks after surgery. 15 Note the positive (arrows) staining of spindled cells. The BMP-4 transgene included an epitope tag (HA epitope, Pharmacia) that facilitated the identification of transgenic BMP-4 molecules. Tissue staining was performed using commercially available polyclonal anti-HA 20 antibodies and standard procedures. Immunostaining was localized only to gap tissues. Control sections included serial sections stained with pre-immune rabbit serum and tissue sections from 13 control osteotomy gaps. instances all controls were negative for peroxidase 25 staining of granulation tissue fibroblasts.

FIG. 7B. Osteotomy gene transfer BMP-4 studies. Shown is the histology of newly formed bone as early as three weeks following gene transfer (FIG. 7A).

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FIG. 8A. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at six weeks' post surgery. 9 and 16 weeks post-op, are presented in FIG. 8B and FIG. 8C, respectively, to demonstrate the orderly growth of new bone in situ over time. This animal, which has been maintained for 23 weeks, has been ambulating normally

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without an external fixator for the past 7 weeks. Similar results have been obtained in a second long term animal (of two) that is now 17 weeks post-op.

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- FIG. 8B. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at nine weeks' post surgery (see FIG. 8A).
- 10 FIG. 8C. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at sixteen weeks' post surgery (see FIG. 8A).
- of the control group that received an osteotomy plus a collagen sponge without DNA of any type. The animal was maintained for 9 weeks following surgery and then sacrificed. Progress of new bone formation in the gap was monitored radiographically and histologically. Shown is a radiograph of the osteotomy gap at 9 weeks. Note the absence of radiodense tissue in the gap.
- PIG. 9B. Shown is a histological section of
 osteotomy gap tissue from the control animal used in FIG
 9A. The section is characterized by the presence of
 granulation tissue fibroblasts and capillaries.
- FIG. 10. PLJ-HPTH1-34 expression construct. A cDNA

 fragment coding for a prepro-hPTH1-34 peptide was
 generated by PCR™ (Hendy et al., 1981) and then ligated
 into a BamHI cloning site in the PLJ retroviral
 expression vector (Wilson et al., 1992). Several
 independent clones with the insert in the coding

 orientation were isolated and characterized.

FIG. 11. Southern analysis of retroviral

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integration in the YZ-15 clone. 10 mg of YZ-15 genomic DNA were digested with KpnI (for which there is a unique site in the vector LTR) and analyzed by Southern blotting. A cDNA fragment that coded for prepro-hPTH1-35 was used as a probe. The positive control for the Southern hybridization conditions was a KpnI digest of genomic DNA from Rat-1 cells infected and selected with the recombinant, replication-defective retrovirus PLJhPTH1-84 (Wilson et al., 1992). KpnI digests of DNA were also prepared from two negative controls: native Rat-1 cells and Rat-1 cells infected and selected with BAG ("BAG cells", (Wilson et al., 1992), a replicationdefective recombinant retrovirus that encodes β galactosidase, which is an irrelevant marker gene in these studies. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2 BAG cells; 3, YZ-15; 4, native Rat-1 cells. DNA sizes (kb) are shown at the left of the figure. As expected, a fragment of the predicted size (e.g., 4.3 kb) is seen only in lane 1 (the positive control) and in lane 3 (YZ-15 DNA).

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FIG. 12. Northern blot analysis of a transduced Rat-1 clone. Poly-A(*)RNA was prepared from the YZ-15 clone and analyzed by Northern blotting as described 25 (Chen et al., 1993). FIG. 12 contains two panels on a single sheet. Poly-A(*) RNA prepared from PLJ-hPTH1-84 cells, BAG cells, and native Rat-1 cells were used as positive and negative controls. Four probes were applied to a single blot following sequential stripping: hPTH1-30 34, β -gal, Neo, and β -actin. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2, BAG cells; 3, YZ-15 cells; 4, native Rat-1 cells. As expected, the hPTH1-34 transcript is seen only in lane 1 (positive control) and in lane 3-4; a Neo transcript is seen in lanes 1-3; a 35 β -gal transcript is seen only in lane 2; and β -actin transcripts are seen in lanes 1-4.

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FIG. 13. Northern analysis of poly-A(*) RNA demonstrating expression of the PTH/PTHrP receptor in osteotomy repair tissue.

- FIG. 14. Overlapping murine cDNA clones representing the LTBP-like (LTBP-3) sequence. A partial representation of restriction sites is shown. N, NcoI; P, PvuII; R, RsaII; B, BamHI; H, HindIII. The numbering system at the bottom assumes that the "A" of the initiator Met codon is nt #1.
 - FIG. 15A. A schematic showing the structure of the murine fibrillin-1 gene product. Structural domains are shown below the diagram. Symbols designating various structural elements are defined in the legend to FIG. 15B.

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- FIG. 15B. A schematic showing the structure of the murine LTBP-like (LTBP-3) molecule. Domains #1-5 are 20 denoted below the diagram. Symbols designate the following structural elements: EGF-CB repeats: open rectangles; TGF-bp repeats: open ovals; Fib motif: open circle; TGF-bp-like repeat: patterned oval; cysteine-rich sequences: patterned rectangles; proline/glycine-rich region: thick curved line, domain #2; proline-rich 25 region, thick curved line, domain #3. Note that symbols designating the signal peptide have been deleted for simplicity. Additionally, the schematic assumes that EGF-like and EGF-CB repeats may extend for several amino 30 acids beyond the C, position.
- FIG. 15C. A schematic showing the structure of human LTBP-1. Domains #1-5 are denoted below the diagram. The symbols designating the structural elements are defined in the legend to FIG. 15B.
 - FIG. 16. Overview of expression of the new LTBP-

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like (LTBP-3) gene during murine development as determined by tissue in situ hybridization. FIG. 16 consists of autoradiograms made by direct exposure of tissue sections to film after hybridization with radiolabeled probes. Day 8.5-9.0 sections contained embryos surrounded by intact membranes, uterine tissues, and the placental disk, cut in random planes. Day 13.5 and 16.5 sections contain isolated whole embryos sectioned in the sagittal plane near or about the midline. Identical conditions were maintained throughout autoradiography and photography, thereby allowing a comparison of the overall strength of hybridization in all tissue sections. The transcript is expressed in connective tissue, mesenchyme, liver, heart and CNS.

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FIG. 17A. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. All photographs in FIG. 17A- FIG. 17D were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the neural tube, brightfield image. 1 cm = 20 mm.

FIG. 17B. Selected microscopic views of mouse

25 LTBP-3 gene expression in day 8.5-9.0 p.c. mouse
developing tissues. Shown is the neural tube, darkfield
image. Note expression by neuroepithelial cells and by
surrounding mesenchyme. 1 cm = 20 mm.

FIG. 17C. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the heart, brightfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. 1 cm = 20 mm.

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FIG. 17D. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse

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developing tissues. Shown is the heart, darkfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. Darkfield photomicrographs were taken after exposure of tissues to photographic emulsion for 2 weeks. In this image and the one shown in FIG. 17B, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. 1 cm = 20 mm.

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FIG. 18A. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. All photographs in FIG. 18A - FIG. 18P were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the cartilage model of developing long bone from lower extremity, brightfield image. Expression by chondrocytes and by perichondrial cells is seen in FIG. 18B. 1 cm = 20 mm.

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FIG. 18B. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the cartilage model of developing long bone from lower extremity, darkfield image. Note expression by chondrocytes and by perichondrial cells. In all darkfield views of FIG. 18, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. Note the absence of spurious hybridization signal in areas of the slide that lack cellular elements. 1 cm = 20 mm.

PIG. 18C. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, brightfield image. 1 cm = 20 mm.

FIG. 18D. Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, brightfield image. 1 cm = 20 mm.

FIG. 18E. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, darkfield image. Note expression by epithelial cells of developing airway and by the surrounding parenchymal cells. 1 cm = 20 mm.

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FIG. 18F. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, darkfield image. Note continuing expression by myocardial cells. 1 cm = 20 mm.

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FIG. 18G. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, brightfield image. 1 cm = 20 mm.

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FIG. 18H. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the intestine, brightfield image. 1 cm = 20 mm.

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FIG. 18I. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, darkfield image. Note expression by acinar epithelial cells. 1 cm = 20 mm.

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FIG. 18J. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is intestine, darkfield image. Note the expression in epithelial and subepithelial cells. 1 cm = 20 mm.

FIG. 18K. Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, brightfield image. 1 cm = 20 mm.

- FIG. 18L. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is skin, brightfield image. 1 cm = 20 mm.
- 10 FIG. 18M. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, darkfield image. Note expression by blastemal cells beneath the kidney capsule, epithelial cells of developing nephrons and tubules, and the interstitial mesenchyme. 1 cm = 20 mm.
- FIG. 18N. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the skin, darkfield image. Note the expression by epidermal, adnexal and dermal cells of developing skin. 1 cm = 20 mm.
- FIG. 180. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the retina, brightfield image. 1 cm = 20 mm.
- FIG. 18P. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the retina, darkfield image. Note expression by retinal epithelial cells and by adjacent connective tissue cells. 1 cm = 20 mm.
- gene by MC3T3-E1 cells. mRNA preparation and Northern blotting were preformed as described in Example XIV. Equal aliquots of total RNA as determined by UV

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spectroscopy were loaded in each lane of the Northern gel. As demonstrated by UV spectroscopy were loaded in each lane of the Northern gel. As demonstrated by methylene blue staining (Sambrook et al., 1989), equal amounts of RNA were transferred to the nylon membrane. The results demonstrate a clear, strong peak in LTBP-3 gene expression by 14 days in culture. Weaker signals denoting LTBP-3 gene expression also can be observed after 5 days and 28 days in culture.

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FIG. 20. Antisera #274 specifically binds LTBP-3 Transfection of 293T cells with a full length mouse LTBP-3 expression plasmid followed by radiolabeling, preparation of medium sample, immunoprecipitation, and 4-18% gradient SDS-PAGE were 15 performed as described in Example XIV. The figure presents a SDS-PAGE autoradiogram of medium samples following a 2 day exposure to film. Lane assignments are as follows: Lane 1, radiolabeled 293T medium (prior to 20 transfection) immunoprecipitated with preimmune serum; Land 2, radiolabeled 293T medium (prior to transfection) immunoprecipitated with antibody #274; Lane 3, radiolabeled 393T medium (following transfection and preincubation with 10 µg of LTBP-3 synthetic peptide 25 cocktail) immunoprecipitated with antibody #274; and Lane 4, radiolabeled 293T medium (following transfection) immunoprecipitated with antibody #274. As indicated by the bar, the full length LTBP-3 molecule migrated at 180-190 kDa.

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FIG. 21. Co-immunoprecipitation of LTBP-3 and TGR- β 1 produced by MC3T3-E1 cells. Aliquots (~10° incorporated CPM) of radiolabeled media produced by MC3T3-E1 cells after 7 days in culture were immunoprecipitated as described in Example XIV. Bars indicate the position of cold molecular weight standards used to estimate molecular weight (Rainbow mix,

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Amersham). Immunoprecipitates were separated using 4%-18% gradient SDS-PAGE and reducing conditions. The figure shows a negative control lane 1 consisting of MC3T3-El medium immunoprecipitated with anti-LTBP-3 antibody #274. Western blotting was performed using the lower portion of the gradient gel and a commercially available antibody to TGF-β1 (Santa Cruz Biotechnology, Inc.). Antibody staining was detected using commercially available reagents and protocols (ECL Western Blotting Reagent, Amersham). MC3T3-El medium was immunoprecipitated with anti-LTBP-2 antibody #274.

FIG. 22A. Radiographic analysis of the type II collagen osteotomy gap three weeks after surgery.

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FIG. 22B. Radiographic analysis of the type I collagen osteotomy gap three weeks after surgery.

FIG. 22C. Histologic analysis of the type II collagen osteotomy shown in FIG. 22A.

FIG. 23A. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Positive (arrows) β -gal cytoplasmic staining is observed in the fracture repair cells.

FIG. 23B. Adenovirus-mediated gene transfer into bone repair/regeneration cells in vivo. Serial section negative control strained with the vehicle of the β -gal antibody plus a cocktail of non-specific rabbit IgG antibodies.

FIG. 23C. Adenovirus-mediated gene transfer into bone repair/regeneration cells in vivo. Osteotomy site was filled with a fibrous collagen implant material soaked in a solution of the replication-defective recombinant adenovirus AdRSVβ-gal (~10¹¹ plaque forming

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units/ml). Note the positive (arrow) β -gal nuclear staining of chondrocytes within the osteotomy site, as demonstrated by immunohistochemistry using a specific anti- β -gal antibody.

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- FIG. 24. The murine BMP-4 amino acid sequence, SEQ ID NO:1. The HA epitope is shown in bold at the extreme carboxy terminus of the sequence.
- 10 FIG. 25. DNA sequence of the murine LTBP-3 gene (SEQ ID NO:2).
 - FIG. 26. Amino acid sequence of the murine LTBP-3 gene product (SEQ ID NO:3).

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- FIG. 27. DNA sequence of the murine LTBP-2 gene (SEQ ID NO:17).
- FIG. 28. Amino acid sequence of the murine LTBP-2 gene product (SEQ ID NO:18).

DESCRIPTION OF THE PREFERRED EMBODIMENT

25 1. Applications of Bone Repair Technology to Human Treatment

The following is a brief discussion of four human conditions to exemplify the variety of diseases and disorders that would benefit from the development of new technology to improve bone repair and healing processes. In addition to the following, several other conditions, such as, for example, vitamin D deficiency; wound healing in general; soft skeletal tissue repair; and cartilage and tendon repair and regeneration, may also benefit from technology concerning the stimulation of bone progenitor cells.

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The first example is the otherwise healthy individual who suffers a fracture. Often, clinical bone fracture is treated by casting to alleviate pain and allow natural repair mechanisms to repair the wound. While there has been progress in the treatment of fracture in recent times, even without considering the various complications that may arise in treating fractured bones, any new procedures to increase bone healing in normal circumstances would represent a great advance.

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A second example which may benefit from new treatment methods is osteogenesis imperfecta (OI). OI encompasses various inherited connective tissue diseases that involve bone and soft connective tissue fragility in humans (Byers and Steiner, 1992; Prockop, 1990). About one child per 5,000-20,000 born is affected with OI and the disease is associated with significant morbidity throughout life. A certain number of deaths also occur, resulting in part from the high propensity for bone fracture and the deformation of abnormal bone after fracture repair (OI types II-IV; Bonadio and Goldstein, 1993). The relevant issue here is quality of life; clearly, the lives of affected individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

OI type I is a mild disorder characterized by bone fracture without deformity, blue sclerae, normal or near normal stature, and autosomal dominant inheritance (Bonadio and Goldstein, 1993). Osteopenia is associated with an increased rate of lone bone fracture upon ambulation (the fracture frequency decreases dramatically at puberty and during young adult life, but increases once again in late middle age). Hearing loss, which often begins in the second or third decade, is a feature of this disease in about half the families and can

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progress despite the general decline in fracture frequency. Dentinogenesis imperfecta is observed in a subset of individuals.

In contrast, OI types II-VI represent a spectrum of more severe disorders associated with a shortened lifespan. OI type II, the perinatal lethal form, is characterized by short stature, a soft calvarium, blue sclerae, fragile skin, a small chest, floppy appearing lower extremities (due to external rotation and abduction of the femurs), fragile tendons and ligaments, bone fracture with severe deformity, and death in the perinatal period due to respiratory insufficiency. Radiographic signs of bone weakness include compression of the femurs, bowing of the tibiae, broad and beaded ribs, and calvarial thinning.

OI type III is characterized by short stature, a triangular facies, severe scoliosis, and bone fracture with moderate deformity. Scoliosis can lead to emphysema and a shortened life-span due to respiratory insufficiency. OI type IV is characterized by normal sclerae, bone fracture with mild to moderate deformity, tooth defects, and a natural history that essentially is intermediate between OI type II and OI type I.

More than 200 OI mutations have been characterized since 1989 (reviewed in Byers and Steiner, 1992; Prockop, 1990). The vast majority occur in the COLIA1 and COLIA2 genes of type I collagen. Most cases of OI type I appear to result from heterozygous mutations in the COLIA1 gene that decrease collagen production but do not alter primary structure, i.e., heterozygous null mutations affecting COLIA1 expression. Most cases of OI types II-IV result from heterozygous mutations in the COLIA1 and COLIA2 genes that alter the structure of collagen.

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A third important example is osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. Risk factors for osteoporosis include increasing age, gender (more females), low bone mass, early menopause, race (Caucasians), low calcium intake, reduced physical activity, genetic factors, environmental factors (including cigarette smoking and abuse of alcohol or caffeine), and deficiencies in neuromuscular control that create a propensity to fall.

More than a million fractures in the USA each year

can be attributed to osteoporosis, and in 1986 alone the
treatment of osteoporosis cost an estimated 7-10 billion
health care dollars. Demographic trends (i.e., the
gradually increasing age of the US population) suggest
that these costs may increase 2-3 fold by the year 2020

if a safe and effective treatment is not found. Clearly,
osteoporosis is a significant health care problem.

Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age. Much of the morbidity and mortality associated with osteoporosis results from immobilization of elderly patients following fracture.

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Current therapies for osteoporosis patients focus on fracture prevention, not fracture repair. This remains an important consideration because of the literature, which clearly states that significant morbidity and mortality are associated with prolonged bed rest in the elderly, particularly those who have suffered hip fractures. Complications of bed rest include blood clots

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and pneumonia. These complications are recognized and measures are usually taken to avoid them, but these measures hardly represent the best approach to therapy. Thus, the osteoporotic patient population would benefit from new therapies designed to strengthen bone and speed up the fracture repair process, thereby getting these people on their feet before the complications arise.

A fourth example is related to bone reconstruction and, specifically, the ability to reconstruct defects in 10 bone tissue that result from traumatic injury; cancer or cancer surgery; birth defect; a developmental error or heritable disorder; or aging. There is a significant orthopaedic need for more stable total joint implants, 15 and cranial and facial bone are particular targets for this type of reconstructive need. The availability of new implant materials, e.g., titanium, has permitted the repair of relatively large defects. Titanium implants provide excellent temporary stability across bony defects. However, experience has shown that a lack of 20 viable bone bridging the defect can result in exposure of the appliance, infection, structural instability and, ultimately, failure to repair the defect.

25 Autologous bone grafts are another possible reconstructive modality, but they have several demonstrated disadvantages in that they must be harvested from a donor site such as iliac crest or rib, they usually provide insufficient bone to completely fill the 30 defect, and the bone that does form is sometimes prone to infection and resorption. Partially purified xenogeneic preparations are not practical for clinical use because microgram quantities are purified from kilograms of bovine bone, making large scale commercial production both costly and impractical. Allografts and 35 demineralized bone preparations are therefore often employed.

Microsurgical transfers of free bone grafts with attached soft tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. However, these techniques are time consuming, have been shown to produce a great deal of morbidity, and can only be used by specially trained individuals. Furthermore, the bone implant is often limited in quantity and is not readily contoured. In the mandible, for example, the majority of patients cannot wear dental appliances using presently accepted techniques (even after continuity is established), and thus gain little improvement in the ability to masticate. Toriumi et al., have written that, "reconstructive surgeons should have at their disposal a bone substitute that would be reliable, biocompatible, easy to use, and long lasting and that would restore mandibular continuity with little associated morbidity. "

In connection with bone reconstruction, specific problem areas for improvement are those concerned with 20 treating large defects, such as created by trauma, birth defects, or particularly, following tumor resection. success of orthopaedic implants, interfaces and artificial joints could conceivably be improved if the 25. surface of the implant, or a functional part of an implant, were to be coated with a bone stimulatory agent. The surface of implants could be coated with one or more appropriate materials in order to promote a more effective interaction with the biological site surrounding the implant and, ideally, to promote tissue 30 repair.

Bone Repair

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Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The

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initiation of new bone formation involves the commitment, clonal expansion, and differentiation of progenitor cells. Once initiated, bone formation is promoted by a variety of polypeptide growth factors, Newly formed bone is then maintained by a series of local and systemic growth and differentiation factors.

The concept of specific bone growth-promoting agents is derived from the work of Huggins and Urist. Huggins 10 et al., 1936, demonstrated that autologous transplantation of canine incisor tooth to skeletal muscle resulted in local new bone formation (Huggins et al., 1936). Urist and colleagues reported that demineralized lyophilized bone segments induced bone formation (Urist, 1965; Urist et al., 1983), a process 15 that involved macrophage chemotaxis; the recruitment of progenitor cells; the formation of granulation tissue, cartilage, and bone; bone remodeling; and marrow differentiation. The initiation of cartilage and bone formation in an extraskeletal site, a process referred to 20 as osteoinduction, has permitted the unequivocal identification of initiators of bone morphogenesis (Urist, 1965; Urist et al., 1983; Sampath et al., 1984; Wang et al., 1990; Cunningham et al., 1992).

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Significant progress has now been made in characterizing the biological agents elaborated by active bone tissue during growth and natural bone healing. Demineralized bone matrix is highly insoluble; Sampath and Reddi (1981) showed that only 3% of the proteins can be extracted using strong combinations of denaturants and detergents. They also showed that the unfractionated demineralized bone extract will initiate bone morphogenesis, a critical observation that led to the purification of "osteoinductive" molecules. Families of proteinaceous osteoinductive factors have now been purified and characterized. They have been variously

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referred to in the literature as bone morphogenetic or morphogenic proteins (BMPs), osteogenic bone inductive proteins or osteogenic proteins (OPs).

3. Bone Repair and Bone Morphogenetic Proteins (BMPs)

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Following their initial purification, several bone morphogenetic protein genes have now been cloned using molecular techniques (Wozney et al., 1988; Rosen et al., 1989; summarized in Alper, 1994). This work has established BMPs as members of the transforming growth factor- β (TGF- β) superfamily based on DNA sequence homologies. Other TGF molecules have also been shown to participate in new bone formation, and TGF- β is regarded as a complex multifunctional regulator of osteoblast function (Centrella et al., 1988; Carrington et al., 1988; Seitz et al., 1992). Indeed, the family of transforming growth factors (TGF- β 1, TGF- β 2, and TGF- β 3) has been proposed as potentially useful in the treatment of bone disease (U.S. Patent 5,125,978, incorporated herein by reference).

The cloning of distinct BMP genes has led to the designation of individual BMP genes and proteins as BMP-1 through BMP-8. BMPs 2-8 are generally thought to be osteogenic (BMP-1 may be a more generalized morphogen; Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). TGFs and BMPs each act on cells via complex, tissue-specific interactions with families of cell surface receptors (Roberts and Sporn, 1989; Paralkar et al., 1991).

Several BMP (or OP) nucleotide sequences and vectors, cultured host cells and polypeptides have been described in the patent literature. For example, U.S. Patents, 4,877,864, 4,968,590 and 5,108,753 all concern

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osteogenic factors. More specifically, BMP-1 is disclosed in U.S. Patent 5,108,922; BMP-2 species, including MBP-2A and BMP-2B, are disclosed in U.S. Patents 5,166,058, 5,013,649, and 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference. Various BMP clones and their activities are particularly described by Wozney et al., (1988; incorporated herein by reference). DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691. Although the BMP terminology is widely used, it may prove to be the case that there is an OP counterpart term for every individual BMP (Alper, 1994).

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4. Bone Repair and Growth Factors and Cytokines

Transforming growth factors (TGFs) have a central role in regulating tissue healing by affecting cell proliferation, gene expression, and matrix protein 20 synthesis (Roberts and Sporn, 1989). While not necessarily a direct effect, Bolander and colleagues have provided evidence that TGF- β 1 and TGF- β 2 can initiate both chondrogenesis and osteogenesis (Joyce et al., 1990; Izumi et al., 1992; Jingushi et al., 1992). In these 25 studies new cartilage and bone formation appeared to be dose dependent (i.e., dependent on the local growth factor concentration). The data also suggested that TGF- β 1 and TGF- β 2 stimulated cell differentiation by a similar mechanism, even though they differed in terms of the ultimate amount of new cartilage and bone that was formed.

Other growth factors/hormones besides TGF and BMP may influence new bone formation following fracture.

Bolander and colleagues injected recombinant acidic fibroblast growth factor into a rat fracture site

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(Jingushi et al., 1990). The major effect of multiple high doses (1.0 mg/50 ml) was a significant increase in cartilage tissue in the fracture gap, while lower doses had no effect. These investigators also used the reverse transcriptase-polymerase chain reaction (PCR^m) technique to demonstrate expression of estrogen receptor transcripts in callus tissue (Boden et al., 1989). These results suggested a role for estrogen in normal fracture repair.

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Horowitz and colleagues have shown that activated osteoblasts will synthesize the cytokine, macrophage colony stimulating factor (Horowitz et al., 1989). The osteotropic agents used in this study included lipopolysaccharide, PTH1-84, PTH1-34, vitamin D and all-trans retinoic acid. This observation has led to the suggestion that osteoblast activation following fracture may lead to the production of cytokines that regulate both hematopoiesis and new bone formation. Various other proteins and polypeptides that have been found to be expressed at high levels in osteogenic cells, such as, e.g., the polypeptide designated Vgr-1 (Lyons et al., 1989), also have potential for use in connection with the present invention.

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5. Bone Repair and Calcium Regulating Hormones

Calcium regulating hormones such as parathyroid hormone (PTH) participate in new bone formation and bone remodeling (Raisz and Kream, 1983). PTH is an 84 amino acid calcium-regulating hormone whose principle function is to raise the Ca² concentration in plasma and extracellular fluid. Studies with the native hormone and with synthetic peptides have demonstrated that the aminoterminus of the molecule (aa 1-34) contains the structural requirements for biological activity (Tregear et al., 1973; Hermann-Erlee et al., 1976; Riond, 1993).

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PTH functions by binding to a specific cell surface receptor that belongs to the G protein-coupled receptor superfamily (Silve et al., 1982; Rizzoli et al., 1983; Juppner et al., 1991).

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Using a retroviral approach, a human full-length PTH gene construct has been introduced into cultured rat fibroblasts to create recombinant PTH-secreting cells. These cells were then transplanted into syngeneic rat recipients that were observed to develop hypercalcemia mediated by the increased serum concentrations of PTH (Wilson et al., 1992). The object of these studies was to create an animal model of primary hyperparathyroidism.

15 PTH has a dual effect on new bone formation, a somewhat confusing aspect of hormone function despite intensive investigation. PTH has been shown to be a potent direct inhibitor of type I collagen production by osteoblasts (Kream et al., 1993). Intact PTH was also 20 shown to stimulate bone resorption in organ culture over 30 years ago, and the hormone is known to increase the number and activity of osteoclasts. Recent studies by Gay and colleagues have demonstrated binding of [125I] PTH(1-84) to osteoclasts in tissue sections and that osteoclasts bind intact PTH in a manner that is both 25 saturable and time- and temperature dependent (Agarwala and Gay, 1992). While these properties are consistent with the presence of PTH/PTHrP receptors on the osteoclast cell surface, this hypothesis is still 30 considered controversial. A more accepted view, perhaps, is that osteoclast activation occurs via an osteoblast signaling mechanism.

On the other hand, osteosclerosis may occur in human patients with primary hyperparathyroidism (Seyle, 1932). It is well known that individuals with hyperparathyroidism do not inexorably lose bone mass, but

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eventually achieve a new bone remodeling steady state after an initial period of net bone loss. Chronic, low dose administration of the amino-terminal fragment of PTH (aa 1-34) also can induce new bone formation according to a time- and dose-dependent schedule (Seyle, 1932; Parsons and Reit, 1974).

Human PTH1-34 has recently been shown to: stimulate DNA synthesis in chick osteoblasts and chondrocytes in culture (van der Plas, 1985; Schluter et al., 1989; 10 Somjen et al., 1990); increase bone cell number in vivo (Malluche et al., 1986); enhance the in vitro growth of chick embryonic cartilage and bone (Kawashima, 1980; Burch and Lebovitz, 1983; Lewinson and Silbermann, 1986; Endo et al., 1980; Klein-Nulend et al., 1990); enhance 15 surface bone formation (both cortical and trabecular bone) in normal and osteogenic animals and in humans with osteoporosis (Reeve et al., 1976; Reeve et al., 1980; Tam et al., 1982; Hefti et al., 1982; Podbesek et al., 1983; Stevenson and Parsons, 1983; Slovik et al., 1986; 20 Gunness-Hey and Hock, 1984; Tada et al., 1988; Spencer et al., 1989; Hock and Fonseca, 1990; Liu and Kalu, 1990; Hock and Gera, 1992; Mitlak et al., 1992; Ejersted et al., 1993); and delay and reverse the catabolic effects of estrogen deprivation on bone mass (Hock et al., 1988; 25 Hori et al., 1988; Gunness-Hey and Hock, 1989; Liu et al., 1991). Evidence of synergistic interactions between hPTH-1-34 and other anabolic molecules has been presented, including insulin-like growth factor, BMP-2, 30 growth hormone, vitamin D, and TGF- β (Slovik et al., 1986; Spencer et al., 1989; Mitlak et al., 1992; Canalis et al., 1989; Linkhart and Mohan, 1989; Seitz et al., 1992; Vukicevic et al., 1989).

35 Anecdotal observation has shown that serum PTH levels may be elevated following bone fracture (Meller et al., 1984; Johnston et al., 1985; Compston et al., 1989;

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Hardy et al., 1993), but the significance of this observation is not understood. There are apparently no reports in the literature concerning attempts to localize either PTH or the PTH/PTHrP receptor in situ in human fracture sites or in experimental models. Furthermore, no attempt has been made to augment bone repair by the exogenous addition of PTH peptides. Although hPTH1-34 is known to function as an anabolic agent for bone, prior to the present invention, much remained to be learned about the role (if any) of PTH during bone regeneration and repair.

6. Protein Administration and Bone Repair

Several studies have been conducted in which preparations of protein growth factors, including BMPs, have been administered to animals in an effort to stimulate bone growth. The results of four such exemplary studies are described blow.

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Toriumi et al., studied the effect of recombinant BMP-2 on the repair of surgically created defects in the mandible of adult dogs (Toriumi et al., 1991). six adult hounds were segregated into three groups following the creation of a 3 cm full thickness mandibular defect: 12 animals received test implants composed of inactive dog bone matrix carrier and human BMP-2, 10 animals received control implants composed of carrier without BMP-2, and BMP-4 animals received no implant. The dogs were euthanized at 2.5-6 months, and the reconstructed segments were analyzed by radiography, histology, histomorphometry, and biomechanical testing. Animals that received test implants were euthanized after 2.5 months because of the presence of well mineralized bone bridging the defect. The new bone allowed these animals to chew a solid diet, and the average bending strength of reconstructed mandibles was 27% of normal

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('normal' in this case represents the unoperated, contralateral hemimandible). In contrast, the implants in the other two groups were non-functional even after 6 months and showed minimal bone formation.

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Yasko et al., published a related study in which the effect of BMP-2 on the repair of segmental defects in the rat femur was examined (Yasko et al., 1992). The study design included a group that received a dose of 1.4 mg of BMP-2, another group that received 11.0 mg of BMP-2, and a control group that received carrier matrix alone. Endochondral bone formation was observed in both groups of animals that received BMP-2. As demonstrated by radiography, histology, and whole bone (torsion) tests of mechanical integrity, the larger dose resulted in functional repair of the 5-mm defect beginning 4.5 weeks after surgery. The lower dose resulted in radiographic and histological evidence of new bone formation, but functional union was not observed even after 9 weeks post surgery. There was also no evidence of bone formation in control animals at this time.

Chen et al., showed that a single application of 25-100 mg of recombinant TGF-β1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). Bone formation began 21 days following the creation of the wound and reached a peak at day 42, as demonstrated by morphological methods. Active bone remodeling was observed beyond this point.

In a related study, Beck et al., demonstrated that a single application of TGF- β l in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991). Bony closure was achieved within 28 days of the application of 200 mg of

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TGF- β l and the rate of healing was shown to be dose dependent.

Studies such as those described above have thus established that exogenous growth factors can be used to stimulate new bone formation/repair/regeneration in vivo. Certain U.S. Patents also concern methods for treating bone defects or inducing bone formation. For example, U.S. Patent 4,877,864 relates to the administration of a therapeutic composition of bone inductive protein to treat cartilage and/or bone defects; U.S. Patent 5,108,753 concerns the use of a device containing a pure osteogenic protein to induce endochondral bone formation and for use in periodontal, dental or craniofacial reconstructive procedures.

However, nowhere in this extensive literature does there appear to be any suggestion that osteogenic genes themselves may be applied to an animal in order to promote bone repair or regeneration. Indeed, even throughout the patent literature that concerns genes encoding various bone stimulatory factors and their in vitro expression in host cells to produce recombinant proteins, there seems to be no mention of the possibility of using nucleic acid transfer in an effort to express an osteogenic gene in bone progenitor cells in vivo or to promote new bone formation in an animal or human subject.

7. Biocompatible Matrices for use in Bone Repair

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There is a considerable amount of work that has been directed to the development of biocompatible matrices for use in medical implants, including those specifically for bone implantation work. In context of the present invention, a matrix may be employed in association with the gene or DNA coding region encoding the osteotropic polypeptide in order to easily deliver the gene to the

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site of bone damage. Such matrices may be formed from a variety of materials presently in use for implanted medical applications.

In certain cases, the matrix may also act as a "biofiller" to provide a structure for the developing bone and cartilage. However, the formation of such a scaffolding structure is not a primary requirement, rather, the main requirements of the matrix are to be biocompatible and to be capable of delivering a nucleic acid segment to a bone cell or bone tissue site.

Matrices that may be used in certain embodiments include non-biodegradable and chemically defined matrices, such as sintered hydroxyapatite, bioglass, aluminates, and other ceramics. The bioceramics may be altered in composition, such as in calcium-aluminatephosphate; and they may be processed to modify particular physical and chemical characteristics, such as pore size, particle size, particle shape, and biodegradability. Certain polymeric matrices may also be employed if desired, these include acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Patents 4,526,909, and 4,563,489, respectively, each incorporated herein by reference. Particular examples of useful polymers are those of orthoesters, anhydrides, propylene-cofumarates, or a polymer of one or more α-hydroxy carboxylic acid monomers, e.g., α -hydroxy acetic acid (glycolic acid) and/or α -hydroxy propionic acid (lactic acid).

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Some of the preferred matrices for use in present purposes are those that are capable of being resorbed into the body. Potential biodegradable matrices for use in bone gene transfer include, for example, PLGA block copolymers, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, and polyanhydrides. Furthermore, biomatrices comprised of

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pure proteins and/or extracellular matrix components may be employed.

The inventors have shown the use of bone or dermal collagenous materials as matrices, as may be prepared from various commercially-available lyophilized collagen preparations, such as those from bovine or rat skin, as well as PLGA block copolymers. Collagen matrices may also be formulated as described in U.S. Patent 4,394,370, incorporated herein by reference, which concerns the use of collagenous matrices as delivery vehicles for osteogenic protein. UltraFiber, as may be obtained from Norian Corp. (Mountain View, CA), is a preferred matrix. Preferred matrices are those formulated with type II collagen, and most preferably, recombinant type II collagen and mineralized type II collagen.

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Further suitable matrices may also be prepared from combinations of materials, such as PLGA block copolymers, 20 which allow for sustained release; hydroxyapatite; or collagen and tricalciumphosphate. Although sufficient sequestration and subsequent delivery of an osteotropic gene is in no way a limitation of the present invention, should it be desired, a porous matrix and gene 25 combination may also be administered to the bone tissue site in combination with an autologous blood clot. The basis for this is that blood clots have previously been employed to increase sequestration of osteogenic proteins for use in bone treatment (U.S. Patent 5,171,579, 30 incorporated herein by reference) and their use in connection with the present invention is by no means excluded (they may even attract growth factors for cytokines).

8. Collagen

Although not previously proposed for use with a nucleic acid molecule, the use of collagen as a pharmaceutical delivery vehicle has been described. The biocompatibility of collagen matrices is well known in the art. U.S. Patents 5,206,028, 5,128,136, 5,081,106, 4,585,797, 4,390,519, and 5,197,977 (all incorporated herein by reference) describe the biocompatibility of collagen-containing matrices in the treatment of skin lesions, use as a wound dressing, and as a means of controlling bleeding. In light of these documents, therefore, there is no question concerning the suitability of applying a collagen preparation to a tissue site of an animal.

U.S. Patent 5,197,977 describes the preparation of a collagen-impregnated vascular graft including drug materials complexed with the collagen to be released slowly from the graft following implant. U.S. Patent 4,538,603 is directed to an occlusive dressing useful for treating skin lesions and a granular material capable of interacting with wound exudate. U.S. Patent 5,162,430 describes a pharmaceutically acceptable, non-immunogenic composition comprising a telopeptide collagen chemically conjugated to a synthetic hydrophilic polymer.

Further documents that one of skill in the art may find useful include U. S. Patents 4,837,285, 4,703,108, 4,409,332, and 4,347,234, each incorporated herein by reference. These references describe the uses of collagen as a non-immunogenic, biodegradable, and bioresorbable binding agent.

The inventors contemplate that collagen from many sources will be useful in the present invention.

Particularly useful are the amino acid sequences of type

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II collagen. Examples of type II collagen are well known in the art. For example, the amino acid sequences of human (Lee et al., 1989), rat (Michaelson et al., 1994), and murine (Ortman et al., 1994) have been determined (SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, respectively).

Although not previously known to be capable of stimulating bone progenitor cells itself, type II

collagen is herein surprisingly shown to possess this property, which thus gives rise to new possibilities for clinical uses.

9. Nucleic Acid Delivery

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The transfer of nucleic acids to mammalian cells has been proposed a method for treating certain diseases or disorders. Nucleic acid transfer or delivery is often referred to as "gene therapy". Initial efforts toward 20 postnatal (somatic) gene therapy relied on indirect means of introducing genes into tissues, e.g., target cells were removed from the body, infected with viral vectors carrying recombinant genes, and implanted into the body. These type of techniques are generally referred to as ex vivo treatment protocols. Direct in vivo gene transfer 25 has recently been achieved with formulations of DNA trapped in liposomes (Ledley et al., 1987); or in proteoliposomes that contain viral envelope receptor proteins (Nicolau et al., 1983); calcium phosphatecoprecipitated DNA (Benvenisty and Reshef, 1986); and DNA 30 coupled to a polylysine-glycoprotein carrier complex (Wu and Wu, 1988). The use of recombinant replicationdefective viral vectors to infect target cells in vivo has also been described (e.g., Seeger et al., 1984).

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In recent years, Wolff et al., demonstrated that direct injection of purified preparations of DNA and RNA

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into murine skeletal muscle resulted in significant reporter gene expression (Wolff et al., 1990). This was an unexpected finding, and the mechanism of gene transfer could not be defined. The authors speculated that muscle cells may be particularly suited to take up and express polynucleotides in vivo or that damage associated with DNA injection may allow transfection to occur.

Wolff et al., suggested several potential applications of the direct injection method, including (a) the treatment of heritable disorders of muscle, (b) the modification of non-muscle disorders through muscle tissue expression of therapeutic transgenes, (c) vaccine development, and (d) a reversible type of gene transfer, in which DNA is administered much like a conventional pharmaceutical treatment. In an elegant study Liu and coworkers recently showed that the direct injection method can be successfully applied to the problem of influenza vaccine development (Ulmer et al., 1993).

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The use of gene transfer to synoviocytes as a means of treating arthritis has also been discussed (Bandara et al., 1992; Roessler et al., 1993). The protocols considered have included both the ex vivo treatment of isolated synoviocytes and their re-introduction into the animal and also direct gene transfer in which suitable vectors are injected into the joint. The transfer of marker genes into synoviocytes has already been demonstrated using both retroviral and adenoviral technology (Bandara et al., 1992; Roessler et al., 1993).

Despite the exclusive emphasis on protein treatment by those working in the field of new bone growth, the present inventors saw that there was great potential for using nucleic acids themselves to promote bone regeneration/repair in vivo. This provides for a more sophisticated type of pharmaceutical delivery. In

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addition to the ease and cost of preparing DNA, it was also reasoned that using DNA transfer rather than peptide transfer would provide many further advantages. For example, DNA transfer allows for the expression or over-expression of integral membrane receptors on the surface of bone regeneration/repair cells, whereas this cannot be done using peptide transfer because the latter (a priori) is an extracellular manipulation. Importantly, DNA transfer also allows for the expression of polypeptides modified in a site-directed fashion with the very minimum of additional work (i.e., straightforward molecular biological manipulation without protein purification) as well as sustained release of therapies delivered by an injectable route.

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The advantages of using DNA are also manifold regarding the development of pharmaceutical products and effective means of delivery. Here, important advantages include the ability to prepare injectable formulations, especially those compositions that exhibit reversible thermal gelation, and the opportunity to combine such injectables with imaging technology during delivery. "Sustained release" is also an important advantage of using DNA, in that the exogenously added DNA continues to direct the production of a protein product following incorporation into a cell. The use of certain matrix-DNA compositions also allows for a more typical "sustained release" phenomenon in that the operative release of DNA from the matrix admixture can also be manipulated.

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The inventors contemplated that both naked DNA and viral-mediate DNA could be employed in an effort to transfer genes to bone progenitor cells. In beginning to study this, the most appropriate animal model had to be employed, that is, one in which the possibilities of using nucleic acids to promote bone repair could be adequately tested in controlled studies.



10. Osteotomy Model

Prior to the present invention, three model systems were available for study in this area, including Mov13 mice, an animal model of OI. Unfortunately, each of the models suffers from significant drawbacks. With the Mov13 mice, first, these mice typically die in young adulthood because of retrovirus-induced leukemia (Schnieke et al., 1983); second, gene transfer studies in Mov13 mice conducted between postnatal weeks 8-16 (i.e., 10 prior to the development of leukemia) may be complicated by a natural adaptation in which a significant amount of new bone is deposited on the periosteal surface (Bonadio et al., 1993); and third, an osteotropic gene transferred into an osteotomy site may synergize with the active 15 retrovirus and make it even more virulent.

Another system is the *in vivo* bone fracture model created by Einhorn and colleagues (Bonnarens and Einhorn, 1984). However, this model is a closed system that would not easily permit initial studies of gene transfer in vivo. The organ culture model developed by Bolander and colleagues (Joyce et al., 1990) was also available, but again, this model is not suitable for studying gene transfer in vivo. Due to the unsuitability of the above models for studying the effects of gene transfer on bone repair and regeneration, the inventors employed a rat osteotomy system, as described below.

30 The important features of the rat osteotomy model are as follows: under general anesthesia, four 1.2 mm diameter pins are screwed into the femoral diaphysis of normal adult Sprague-Dawley rats. A surgical template ensures parallel placement of the pins. An external fixator is then secured on the pins, and a 2 mm, or 5 mm, segmental defect is created in the central diaphysis with a Hall micro 100 oscillating saw. A biodegradable

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implant material, soaked in a solution of plasmid DNA, other genetic construct or recombinant virus preparation, is then placed in the intramedullary canal and the defect is closed (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

New bone formation can be detected as early as three weeks later in the 2 mm gap, although up to 9 weeks is generally allowed for new bone formation to occur. The fixator provided the necessary stability, and there were no limitations on animal ambulation. The surgical protocol has been successfully performed on 21/21 animals to date. None of these animals have died. Assays of new bone formation are performed after sacrifice, except plain film radiography, which is performed weekly from the time of surgery to sacrifice.

Previous studies in Sprague-Dawley rats have shown that the 5 mm osteotomy gap will heal as a fibrous non-union, whereas a gap of less than 3 mm, (such as the 2 mm gap routinely employed in the studies described herein) will heal by primary bone formation. Studies using the 5 mm gap thus allow a determination of whether transgene expression can stimulate new bone formation when fibrous tissue healing normally is expected. On the other hand, studies with the 2 mm gap allow a determination of whether transgene expression can speed up natural primary bone healing. Controls were also performed in which animals received no DNA (FIG. 9A and FIG. 9B).

11. Gene Transfer Promotes Bone Repair In Vivo

The present inventors surprisingly found that gene transfer into bone progenitor cells in vivo (i.e., cells in the regenerating tissue in the osteotomy gap) could be readily achieved. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous

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collagen implant material soaked in a solution of DNA shortly before being placed in the site in which one desires to promote bone growth. As the studies presented herein show, the implant material facilitates the uptake of exogenous plasmid constructs by cells (in the osteotomy gap) which clearly participate in bone regeneration/repair. The transgenes, following cellular uptake, direct the expression of recombinant polypeptides, as evidenced by the in vivo expression of functional marker gene products.

Further studies are presented herein demonstrating that the transfer of an osteotropic gene results in cellular expression of a recombinant osteotropic molecule, which expression is directly associated with 15 stimulation of new bone formation. After considering a relatively large number of candidate genes, a gene transfer vector coding for a fragment of human parathyroid hormone (hPTH1-34) was chosen for the inventors' initial studies. Several factors were 20 considered in making this choice: (a), recombinant hPTH1-34 peptides can be discriminated from any endogenous rat hormone present in osteotomy tissues; (b), hPTH1-34 peptides will stimulate new bone formation in Sprague-Dawley rats, indicating that the human peptide 25 can efficiently bind the PTH/PTHrP receptor on the rat osteoblast cell surface; and (c), there is only one PTH/PTHrP receptor, the gene for this receptor has been cloned, and cDNA probes to the receptor are available.

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Thus, in terms of understanding the mechanism of action of the transgene on new bone formation in vivo, . the inventors reasoned it most straightforward to correlate the expression of recombinant hPTH1-34 peptide and its receptor with new bone formation in the rat osteotomy model. Of course, following these initial studies, it is contemplated that any one of a wide

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variety of genes may be employed in connection with the bone gene transfer embodiments of the present invention.

Previous studies have indicated that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously. Despite the fact that an anabolic effect would still be expected with continuous dosing, as documented by the studies of Parsons and coworkers (Tam et al., 1982; Spencer et al., 1989), there was a concern that the PLJ-hPTH1-34 transgene may not function very effectively as transfected cells would be expected to express recombinant hPTH1-34 molecules in a constitutive manner. The finding that transfection and expression of the LPH-hPTH1-34 transgene did effectively stimulate bone formation in the rat osteotomy model was therefore an important result.

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As the osteotomy site in this model is highly vascularized, one possible complication of the studies with the PLJ-hPTH1-34 transgene is the secretion of recombinant human PTH from the osteotomy site with consequent hypercalcemia and (potentially) animal death. Weekly serum calcium levels should therefore be determined when using this transgene. The fact that no evidence of disturbed serum calcium levels has been found in this work is therefore a further encouraging finding.

These studies complement others by the inventors in which direct gene transfer was employed to introduce genes into Achilles' tendon and cruciate ligament, as described in Example XI.

Various immediate applications for using nucleic acid delivery in connection with bone disorders became apparent to the inventors following these surprising findings. The direct transfer of an osteotropic gene to promote fracture repair in clinical orthopaedic practice

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is just one use. Other important aspects of this technology include the use of gene transfer to treat patients with "weak bones", such as in diseases like osteoporosis; to improve poor healing which may arise for unknown reasons, e.g., fibrous non-union; to promote implant integration and the function of artificial joints; to stimulate healing of other skeletal tissues such as Achilles' tendon; and as an adjuvant to repair large defects. In all such embodiments, DNA is being used as a direct pharmaceutical agent.

12. Biological Functional Equivalents

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As mentioned above, modification and changes may be

made in the structure of an osteotropic gene and still
obtain a functional molecule that encodes a protein or
polypeptide with desirable characteristics. The
following is a discussion based upon changing the amino
acids of a protein to create an equivalent, or even an

improved, second-generation molecule. The amino acid
changes may be achieved by changing the codons of the DNA
sequence, according to the following codon table:

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Table 1

Amino Acids			Code	ns				
Alanine .	Ala	Ą	GCA	GCC	GCG	GCU		
Cysteine	Сув	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	טטט				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	н	CAC	CAÚ				
Isoleucine	Ile	1	AUA	AUC	AUU			
Lysine	Lys	ĸ	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	coo
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	DAA				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	cgu
Serine	Ser	s	AGC	AGU	UCA	UCC	UCG	ບຕັນ
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	v	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG			,		
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted 25 for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a 30 protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by 35 the inventors that various changes may be made in the DNA sequences of osteotropic genes without appreciable loss of their biological utility or activity.

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In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the

substitution of like amino acids can be made effectively
on the basis of hydrophilicity. U.S. Patent 4,554,101,
incorporated herein by reference, states that the

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greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

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As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

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It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

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13. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a

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double stranded vector which includes within its sequence a DNA sequence which encodes the desired osteotropic protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as E. coli polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as E. coli cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

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The preparation of sequence variants of the selected osteotropic gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of osteotropic genes may be obtained. For example, recombinant vectors encoding the desired osteotropic gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

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14. Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, e.g., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

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As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimyde and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity
of a particular immunogen composition can be enhanced by
the use of non-specific stimulators of the immune
response, known as adjuvants. Exemplary and preferred

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adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

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The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

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MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified LTBP-3 protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred

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as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a 10 rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 107 to 2 X 108 lymphocytes.

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The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

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Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and

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4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

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Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter et al. (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at 25 low frequencies, about 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in 30 a selective medium. The selective medium is generally one that contains an agent that blocks the de novo synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and 35 methotrexate block de novo synthesis of both purines and pyrimidines, whereas azaserine blocks only purine

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synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

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The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal

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antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured in vitro, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

15. LTBP-3

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Other aspects of the present invention concern isolated DNA segments and recombinant vectors encoding 15 LTBP-3, and the creation and use of recombinant host cells through the application of DNA technology, that express LTBP-3 gene products. As such, the invention concerns DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid 20 sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3. These DNA segments are represented by those that include a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2 (FIG. 25). Compositions that include a 25 purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3 (FIG. 26) are also encompassed by the invention.

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The TGF- β s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF- β , two chains of nascent pro-TGF- β associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer.

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Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 1992). During biosynthesis the mature $TGF-\beta$ dimer is cleaved from the propeptide dimer. TGF- β latency results in part from the non-covalent association of propeptide and mature TGF- β dimers (Pircher et al., 1984, 1986; Wakefield et al., 1987; Millan et al., 1992; Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein 10 (LAP), and LAP plus the disulfide-bonded TGF- β dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature $TGF-\beta$. The mechanism of activation of the latent complex is thought to be one of 15 the most important steps governing $TGF-\beta$ effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent 20 growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF- β binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell 25 types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990). 30 Latent TGF- β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- β , but rather it is linked by a disulfide bond to LAP.

Regarding the novel protein LTBP-3, the present invention concerns DNA segments, that can be isolated from virtually any mammalian source, that are free from total genomic DNA and that encode proteins having LTBP-3-like activity. DNA segments encoding LTBP-3-like species may prove to encode proteins, polypeptides, subunits, functional domains, and the like.

As used herein, the term "DNA segment" refers to a
DNA molecule that has been isolated free of total genomic
DNA of a particular species. Therefore, a DNA segment
encoding LTBP-3 refers to a DNA segment that contains
LTBP-3 coding sequences yet is isolated away from, or
purified free from, total genomic DNA of the species from
which the DNA segment is obtained. Included within the
term "DNA segment", are DNA segments and smaller
fragments of such segments, and also recombinant vectors,
including, for example, plasmids, cosmids, phagemids,
20 phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified LTBP-3 gene refers to a DNA segment including LTBP-3 coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding LTBP-3, forms the significant part of the

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coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns
isolated DNA segments and recombinant vectors
incorporating DNA sequences that encode an LTBP-3 species
that includes within its amino acid sequence an amino
acid sequence essentially as set forth in SEQ ID NO:3.
In other particular embodiments, the invention concerns
isolated DNA segments and recombinant vectors
incorporating DNA sequences that include within their
sequence a nucleotide sequence essentially as set forth
in SEQ ID NO:2.

20 The term "a sequence essentially as set forth in SEQ ID NO:3" means that the sequence substantially corresponds to a portion of SEQ ID NO:3 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids 25 of SEQ ID NO:3. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein (for example, see section 7, preferred embodiments). Accordingly, sequences that have between about 70% and about 80%; or more preferably, 30 between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:3 will be sequences that are "essentially as set forth in SEQ ID NO:3".

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In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that

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include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:2. The term "essentially as set forth in SEQ ID NO:2" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:2. Again, DNA segments that encode proteins exhibiting LTBP-3-like activity will be most preferred.

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It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various noncoding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

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Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:2. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:2,

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under relatively stringent conditions such as those described herein.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch identical to or complementary to SEO ID NO:2, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

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It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

35 It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3. Recombinant

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vectors and isolated DNA segments may therefore variously include the LTBP-3 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include LTBP-3-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

10 The DNA segments of the present invention encompass biologically functional equivalent LTBP-3 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, 15 functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by 20 man may be introduced through the application of sitedirected mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the 25 molecular level.

If desired, one may also prepare fusion proteins and peptides, e.g., where the LTBP-3 coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding

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portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a LTBP-3 gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

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In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an LTBP-3 gene in its natural environment. Such promoters may include LTBP-3 promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited

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to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology) (see Example XVI herein).

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In connection with expression embodiments to prepare recombinant LTBP-3 proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire LTBP-3 protein or functional domains, subunits, etc. being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of LTBP-3 peptides or epitopic core regions, such as may be used to generate anti-LTBP-3 antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 15 to about 50 amino acids in length, or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful.

The LTBP-3 gene and DNA segments may also be used in connection with somatic expression in an animal or in the creation of a transgenic animal. Again, in such embodiments, the use of a recombinant vector that directs the expression of the full length or active LTBP-3 protein is particularly contemplated.

In addition to their use in directing the expression of the LTBP-3 protein, the nucleic acid sequences disclosed herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous sequence of SEQ ID NO:2 will find particular utility.

35 Longer contiguous identical or complementary sequences, e.g., those of about 20, 30, 40, 50, 100, 200, 500, 1000

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(including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

5 The ability of such nucleic acid probes to specifically hybridize to LTBP-3-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to SEQ ID NO:2, are particularly contemplated as hybridization probes for use in, e.g., Southern and Northern blotting. This would allow LTBP-3 structural or regulatory genes to be 20 analyzed, both in diverse cell types and also in various mammalian cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments 25 will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 nucleotides, but larger contiguous complementarity 30 stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 10-14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 10 bases in length are generally preferred,

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though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having genecomplementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequence set forth in SEQ ID NO:2 and to select any continuous portion of the sequence, from about 10-14 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence.

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The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from 20 within SEQ ID NO:2 may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, e.g., by mechanical shearing or by restriction enzyme 25 digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such 30 as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA 35 techniques generally known to those of skill in the art of molecular biology.

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Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of LTBP-3 gene or cDNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating LTBP-3 genes.

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Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one 20 seeks to isolate LTBP-3-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ 25 conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from 20°C to 55°C. Crosshybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated 30 that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions 35 can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

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In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents 20 in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. fixed, single-stranded nucleic acid is then subjected to 25 specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size 30 of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

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The following examples are included to demonstrate preferred embodiments of the invention. It should be

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appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

ANIMAL MODEL FOR ASSESSING NEW BONE FORMATION

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As various animal models were not suitable for studying the effects of nucleic acid transfer on bone formation, the inventors employed the following model system. The important features of the rat osteotomy model are as described in the following protocol (which is generally completed in 25-35 minutes).

The osteotomy was performed on one femur per animal. Right to left differences have not been apparent, but such differences are monitored in these studies, since the limb receiving the osteotomy is randomized.

After pre-operative preparation (i.e., shaving and Betadine® scrub), adult male Sprague Dawley rats (~500 gm, retired male breeders) were anesthetized using a 3% halothane 97% oxygen mixture (700 ml/min. flow rate). A lateral approach to the femur was made on one limb. Utilizing specially designed surgical guides, four 1.2-mm diameter pins were screwed into the diaphysis after predrilling with a high speed precision bit. A surgical template ensured precise and parallel placement of the pins. The order of pin placement was always the same:

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outer proximal first and then outer distal, inner proximal and inner distal (with "outer" and "inner" referring to the distance from the hip joint). Pin placement in the center of the femur was ensured by fluoroscopic imaging during pin placement. The external fixator was secured on the pins and a t mm or 2 mm segmental defect was created in the central diaphysis through an incision using a Hall Micro 100 Oscillating saw (#5053-60 Hall surgical blades) under constant irrigation. Other than the size of the segmental defect, there is no difference between the 5 mm and 2 mm osteotomy protocols (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

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The contents of the osteotomy site were irrigated with sterile saline and the fibrous collagen implant material, previously soaked in a solution of plasmid DNA or other DNA construct, if appropriate, was placed in 20 The wound was then closed in layers. fixator provided the necessary stability no limitations on animal ambulation existed, and other supports were not required. The surgical protocol has been successfully performed on 53 animals to date, including 35 controls (Table 2 and FIG. 24). None of these animals have died 25 and no significant adverse effects have been observed, other than complications that might be associated with surgical fracture repair. Minor complications that were experienced include 1 animal that developed a post-30 operative osteomyelitis and 1 animal in which 2/4 pins loosened as a consequence of post-operative bone fracture.

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EXAMPLE II

IMPLANT MATERIAL FOR USE IN BONE GENE TRANSFER

Various implant materials may be used for transferring genes into the site of bone repair and/or regeneration in vivo. These materials are soaked a solution containing the DNA or gene that is to be transferred to the bone regrowth site. Alternatively, DNA may be incorporated into the matrix as a preferred method of making.

One particular example of a suitable material is fibrous collagen, which may be lyophilized following extraction and partial purification from tissue and then sterilized. A particularly preferred collagen is the fibrous collagen implant material termed UltraFiber, as may be obtained from Norian Corp., (Mountain View, CA). Detailed descriptions of the composition and use of UltraFiber are provided in Gunasekaran et al., (1993a, b; each incorporated herein by reference).

A more particularly preferred collagen is type II collagen, with most particularly preferred collagen being either recombinant type II collagen, or mineralized type II collagen. Prior to placement in osteotomy sites, implant materials are soaked in solutions of DNA (or virus) under sterile conditions. The soaking may be for any appropriate and convenient period, e.g., from 6 minutes to over-night. The DNA (e.g., plasmid) solution will be a sterile aqueous solution, such as sterile water or an acceptable buffer, with the concentration generally being about 0.5 - 1.0 mg/ml. Currently preferred plasmids are those such as pGL2 (Promega), pSV40β-gal, pAd.CMVlacZ, and pLJ.

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EXAMPLE III PARATHYROID HORMONE GENE CONSTRUCTS

The active fragment of the human parathyroid hormone gene (hPTH1-34) was chosen as the first of the osteotropic genes to be incorporated into an expression vector for use in gene transfer to promote new bone formation in the rat osteotomy model.

The inventors chose to construct the hPTH1-34 transgene in the pLJ expression vector (FIG. 10), since this vector was appropriate for studies of transgene function both in vitro and in vivo. A schematic of the PLJ-hPTH1-34 transgene is shown in FIG. 10. The DNA and amino acid sequences of the hPTH1-34 are well known, e.g., see Hendy et al., (1981, incorporated herein by reference). To insert the transgene into the PLJ expression vector PCR™ of a full-length PTH recombinant clone was employed, followed by standard molecular biological manipulation.

A retroviral stock was then generated following $CaPO_4$ -mediated transfection of ϕ crip cells with the PLJ-hPTH1-34 construct, all according to standard protocols (Sambrook et al., 1989). Independent transduced Rat-1 clones were obtained by standard infection and selection procedures (Sambrook et al., 1989).

One clone (YZ-15) was analyzed by Southern analysis,

demonstrating that the PLJ-hPTH1-34 transgene had stably
integrated into the Rat-1 genome (FIG. 11). A Northern
analysis was next performed to show that the YZ-15 clone
expressed the PLJ-hPTH1-34 transgene, as evidenced by the
presence of specific PLJ-hPTH1-34 transcripts (FIG. 12).

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EXAMPLE IV

PARATHYROID HORMONE POLYPEPTIDE EXPRESSION AND ACTIVITY

A sensitive and specific radioimmunoassay was

performed to demonstrate that the YZ-15 cells expressed
and secreted a recombinant hPTH1-34 molecule (Table 2).

The radioimmunoassay was performed on media from
transduced Rat-1 clones. To quantify secretion of the
recombinant hPTH-1-34 peptide produced by YZ-15 cells,

the culture medium from one 100 mm confluent dish was
collected over a 24 hour period and assayed with the NH2terminal hPTH RIA kit (Nichols Institute Diagnostics)
according to the manufacturer's protocol. PLJ-hPTH1-87
cells and BAG cells served as positive and negative
controls, respectively.

Protein concentrations in Table 2 are expressed as the average of three assays plus the standard deviation (in parenthesis). The concentration of the 1-34 and full length (1-84) peptides was determined relative to a standard curve generated with commercially available reagents (Nichols Institute Diagnostics).

Table 2

25	CELL	LINE	

CELL LINES	PTH (pg/ml)		
YZ-15	247 (± 38)		
PLJ-hPTH1-84	2616 (± 372)		
BAG	13 (± 3)		

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As shown in Table 2, PTH expression was detected in both YZ-15 cells and PLJ-hPTH1-84 cells. BAG cells produced no detectable PTH and served as a baseline for the RIA. These results demonstrate that YZ-15 cells expressed recombinant hPTH1-34 protein.

The recombinant hPTH1-34 molecule was added to rat

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osteosarcoma cells and a cAMP response assay conducted in order to determine whether the secreted molecule had biological activity. Unconcentrated media was collected from YZ-15 cells, PLJ-hPTH1-84 cells, and BAG cells and was used to treat ROS17/2.8 cells for 10 minutes, as described (Majmudar et al., 1991). cAMP was then extracted from treated cells and quantified by RIA (Table 3). The amount of cAMP shown is the average of three assays. The standard deviation of the mean is shown in parenthesis.

Table 3

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CELL LINES	cAMP (pmol)		
YZ-15	20.3	(± 0.25)	
PLJ-hPTH184	88.5	(± 4.50)	
BAG	7.6	(± 0.30)	

A cAMP response was induced by the recombinant PTH
secreted by the YZ-15 cells and by PLJ-hPTH1-84 cells.
BAG cells produced no PTH and served as the baseline for
the cAMP assay. These results provide direct in vitro
evidence that the PLJ-hPTH1-34 transgene directs the
expression and secretion of a functional osteotropic
agent.

EXAMPLE V BONE MORPHOGENETIC PROTEIN (BMP) GENE CONSTRUCTS

- The murine bone morphogenetic protein-4 (BMP-4) was chosen as the next of the osteotropic genes to be incorporated into an expression vector for use in promoting bone repair and regeneration.
- A full length murine BMP-4 cDNA was generated by screening a murine 3T3 cell cDNA library (Stratagene). The human sequence for BMP-4 is well known to those of

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skill in the art and has been deposited in Genbank. Degenerate oligonucleotide primers were prepared and employed in a standard PCR^{∞} to obtain a murine cDNA sequence.

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The ends of the cDNA clone were further modified using the polymerase chain reaction so that the full length cDNA (5'→3' direction) encodes the natural murine initiator Met codon, the full length murine coding sequence, a 9 amino acid tag (known as the HA epitope), and the natural murine stop codon. The amino acid sequence encoded by the murine BMP-4 transgene is shown in FIG. 24; this entire sequence, including the tag, is represented by SEQ ID NO:1.

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Placement of the HA epitope at the extreme carboxy terminus should not interfere with the function of the recombinant molecule sequence in vitro or in vivo. The advantage of the epitope is for utilization in immunohistochemical methods to specifically identify the recombinant murine BMP-4 molecule in osteotomy tissues in vivo, e.g., the epitope can be identified using a commercially available monoclonal antibody (Boehringer-Mannheim), as described herein.

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Studies to demonstrate that the murine BMP-4 transgene codes for a functional osteotropic agent include, for example, (a) transfection of COS cells and immunoprecipitation of a protein band of the correct size using a monoclonal anti-HA antibody (Boehringer-Mannheim); and (b) a quantitative in vivo bone induction bioassay (Sampath and Reddi, 1981) that involves implanting proteins from the medium of transfected COS cells beneath the skin of male rats and scoring for new bone formation in the ectopic site.

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EXAMPLE VI

DETECTION OF MRNA BY TISSUE IN SITU HYBRIDIZATION

The following technique describes the detection of

mRNA in tissue obtained from the site of bone
regeneration. This may be useful for detecting
expression of the transgene mRNA itself, and also in
detecting expression of hormone or growth factor
receptors or other molecules. This method may be used in
place of, or in addition to, Northern analyses, such as
those described in FIG. 13.

DNA from a plasmid containing the gene for which mRNA is to be detected is linearized, extracted, and 15 precipitated with ethanol. Sense and antisense transcripts are generated from 1 mg template with T3 and T7 polymerases, e.g., in the presence of [35S] UTP at >6 mCi/ml (Amersham Corp., >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining in vitro transcription reagents provided in a kit (SureSite, 20 Novagen Inc.). After transcription at 37°C for 1 hour, DNA templates are removed by a 15 minute digestion at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes are hydrolyzed to an average final length of 150 bp by incubating in 40 mM 25 NaHCO3, 60 mM Na2CO3, 80 mM DTT at 60°C, according to previously determined formula. Hydrolysis is terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to 0.09 M and 0.005% (v/v), respectively, and the probes are then ethanol precipitated, dissolved in 30 0.1 M DTT, counted, and stored at -20°C until use.

RNase precautions are taken in all stages of slide preparation. Bouins fixed, paraffin embedded tissue sections are heated to 65°C for 10 minutes, deparaffinized in 3 changes of xylene for 5 minutes, and rehydrated in a descending ethanol series, ending in

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phosphate-buffered saline (PBS). Slides will be soaked in 0.2 N HCl for 5 min., rinsed in PBS, digested with 0.0002% proteinase K in PBS for 30 minutes at 37°C and rinsed briefly with DEPC-treated water. equilibrating for 3 minutes in 0.1 M triethanolamine-HCl (TEA-HCl), pH 8.0, sections are acetylated in 0.25% (v/v) acetic anhydride in 0.1 M TEA-HCl for 10 minutes at room temperature, rinsed in PBS, and dehydrated in an ascending ethanol series. Each section receives 100-200 10 ml prehybridization solution (0.5 mg/ml denatured RNasefree tRNA (Boehringer-Mannheim), 10 mM DTT, 5 mg/ml denatured, sulfurylated salmon sperm DNA, 50% formamide, 10% dextran sulfate, 300 mM NaCl, 1X RNase-free Denhardt's solution (made with RNase-free bovine serum albumin, Sigma), 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and 15 then incubate on a 50°C slide warmer in a humidified enclosure for 2 hours. The sulfurylated salmon-sperm DNA blocking reagent is used in both prehybridization and hybridization solutions to help reduce nonspecific 20 binding to tissue by 35SH groups on the probe. It is prepared by labeling RNase-free salmon sperm DNA (Sigma) with non-radioactive α -thio-dCTP and α -thio-dATP (Amersham) in a standard random oligonucleotide-primed DNA labeling reaction. Excess prehybridization solution 25 is removed with a brief rinse in 4X SSC before application of probe.

Riboprobes, fresh tRNA and sulfurylated salmon sperm DNA will be denatured for 10 minutes at 70°C, and chilled on ice. Hybridization solution, identical to prehybridization solution except with denatured probe added to 5 x 10° CPM/ml, is applied and slides incubated at 50°C overnight in sealed humidified chambers on a slide warmer. Sense and antisense probes are applied to serial sections. Slides are rinsed 3 times in 4X SSC, washed with 2X SSC, 1 mM DTT for 30 min. at 50°C, digested with RNase A (20 mg/ml RNase A, 0.5 M NaCl, 10

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mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min. at 37°C, and rinsed briefly with 2X SSC, 1 mM DTT. Three additional washes are performed, each at 50°C for 30 minutes: once in 2X SSC, 50% formamide, 1 mM DTT, and twice in 1X SSC, 0.13% (w/v) sodium pyrophosphate, 1 mM DTT.

Slides are dehydrated in an ascending ethanol series (with supplementation of the dilute ethanols (50% and 70%) with SSC and DTT to 0.1% and 1 mM, respectively). Slides are exposed to %X-ray film for 20-60 hours to visualize overall hybridization patterns, dipped in autoradiographic emulsion (Kodak NTB-2, diluted to 50% with 0.3 M ammonium acetate), slowly dried for 2 hours, and exposed (4°C) for periods ranging from 8 days to 8 weeks. After developing emulsion, sections are counter strained with hematoxylin and eosin, dehydrated, and mounted with xylene-based medium. The hybridization signal is visualized under darkfield microscopy.

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The above in situ hybridization protocol may be used, for example, in detecting the temporal and spatial pattern of PTH/PTHrP receptor expression. A suitable rat PTH/PTHrP receptor cDNA probe (R15B) is one that consists of a 1810 bp region encoding the full length rat bone PTH/PTHrP receptor (Abou-Samra et al., 1992). The cDNA fragment is subcloned into pcDNA 1 (Invitrogen Corp., San Diego, CA) and is cut out using XbaI and BamHI. probe has provided positive signals for northern blot 30 analysis of rat, murine, and human osteoblastic cell lines, rat primary calvarial cells, and murine bone tissue. The pcDNA I plasmid contains a T7 and SP6 promoter that facilitate the generation of cRNA probes for in situ hybridization. The full length transcript has been used to detect PTH/PTHrP receptor in sections of 35 bone (Lee et al., 1994). The PTHrP cDNA probe (Yasuda et al., 1989) is a 400 bp subcloned fragment in

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pBluescript (Stratagene). This probe has been used for in situ hybridization, generating an antisense cRNA probe using BamHI cleavage and the T3 primer and a sense cRNA probe using EcoRI cleavage and the T7 primer.

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EXAMPLE VII

IN VIVO PROTEIN DETECTION FOLLOWING TRANSGENE EXPRESSION

1. β-galactosidase Transgene

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Bacterial β -galactosidase can be detected immunohistochemically. Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the bacterial β -galactosidase protein.

For immunohistochemistry, cross-Sections (2-3 mm 20 thick) were transferred to poly-L-Lysine coated microscope slides and fixed in acetone at 0°C for at least 20 min. Sections were rehydrated in PBS. Endogenous peroxidase activity was quenched by immersion of tissue sections in 0.1% hydrogen peroxide (in 95% 25 methanol) at room temperature for 10 min, and quenched sections were washed 3x in PBS. In some cases, sectioned calvariae were demineralized by immersion in 4% EDTA, 5% polyvinyl pyrrolidone, and 7% sucrose, pH 7.4, for 24 h at 4°C. Demineralized sections were washed 3x before application for antibodies. Primary antibodies were used 30 without dilution in the form of hybridoma supernatant. Purified antibodies were applied to tissue sections at a concentration of 5 mg/ml. Primary antibodies were detected with biotinylated rabbit antimouse IgG and 35 peroxidase conjugated streptavidin (Zymed Histostain-SPkit). After peroxidase staining, sections were counterstained with hematoxylin.

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Bacterial β -gal can also be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

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2. Luciferase Transgene

Luciferase can be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

3. PTH Transgenes

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Recombinant PTH, such as hPTH1-34 peptide, is assayed in homogenates of osteotomy gap tissue, for example, using two commercially available radioimmunoassay kits according to the manufacturer's protocols (Nichols Institute Diagnostics, San Juan Capistrano, CA).

One kit is the Intact PTH-Parathyroid Hormone 100T Kit. This radioimmunoassay utilizes an antibody to the carboxy terminus of the intact hormone, and thus is used to measure endogenous levels of hormone in gap osteotomy tissue. This assay may be used to establish a baseline value PTH expression in the rat osteotomy model.

The second kit is a two-site immunoradiometric kit for the measurement of rat PTH. This kit uses affinity purified antibodies specific for the amino terminus of the intact rat hormone (PTH1-34) and thus will measure endogenous PTH production as well as the recombinant protein. Previous studies have shown that these antibodies cross-react with human PTH and thus are able to recognize recombinant molecules in vivo.

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Values obtained with kit #1 (antibody to the carboxy terminus) are subtracted from values obtained with kit #2 (antibody to the amino terminus) to obtain an accurate and sensitive measurements. The level of recombinant peptide is thus correlated with the degree of new bone formation.

4. BMP Transgene

10 Preferably, BMP proteins, such as the murine BMP-4
transgene peptide product, are detected
immunohistochemically using a specific antibody that
recognizes the HA epitope (Majmudar et al., 1991), such
as the monoclonal antibody available from Boehringer15 Mannheim. Antibodies to BMP proteins themselves may also
be used. Such antibodies, along with various immunoassay
methods, are described in U.S. Patent 4,857,456,
incorporated herein by reference.

Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the recombinant murine BMP-4 molecule.

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EXAMPLE VIII

DIRECT GENE TRANSFER INTO REGENERATING BONE IN VIVO

To assess the feasibility of direct gene transfer into regenerating bone in vivo, marker gene transfer into cells in the rat osteotomy model was employed. These studies involved two marker genes: bacterial β -galactosidase and insect luciferase.

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Aliquots of a fibrous collagen implant material were soaked in solutions of pure marker gene DNA. The implant

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materials were then placed in the osteotomy site, and their expression determined as described above.

It was found that both marker genes were successfully transferred and expressed, without any failures, as demonstrated by substrate utilization assays (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C and FIG. 6D). Since mammalian cells do not normally synthesize either marker gene product, this provides direct evidence that osteotomy repair cells were transfected in vivo and then expressed the β -galactosidase and luciferase transgenes as a functional enzymes.

EXAMPLE IX

15 ADENOVIRAL GENE TRANSFER INTO REGENERATING BONE IN VIVO

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One of the alternative methods to achieve in vivo gene transfer into regenerating bone is to utilize an adenovirus-mediated transfer event. Successful adenoviral gene transfer of a marker gene construct into bone repair cells in the rat osteotomy model has been achieved (FIG. 23A, FIG. 23B, and FIG. 23C).

The inventors employed the adenoviral vector pAd.

CMVlacZ, which is an example of a replication-defective adenoviral vector which can replicate in permissive cells (Stratford-Perricaudet et al., 1992). In pAd.CMVlacZ, the early enhancer/promoter of the cytomegalovirus (CMV) is used to drive transcription of lacZ with an SV40 polyadenylation sequence cloned downstream from this reporter (Davidson et al., 1993).

The vector pAd.RSV4 is also utilized by the inventors. This vector essentially has the same backbone as pAdCMVlacZ, however the CMV promoter and the single BglII cloning site have been replaced in a cassette-like fashion with BglII fragment that consists of an RSV

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promoter, a multiple cloning site, and a poly(A*) site. The greater flexibility of this vector is contemplated to be useful in subcloning osteotropic genes, such as the hPTH1-34 cDNA fragment, for use in further studies.

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To generate recombinant PTH adenovirus, a 100-mm dish of 293 cells is transfected using calcium phosphate with 20 mg of a plasmid construct, e.g., the plasmid containing the hPTH1-34 insert linearized with WheI, plus 2 mg of wild type adenovirus DNA digested with XbaI and 10 The adenovirus DNA is derived from adenovirus type 5, which contains only a single XbaI and ClaI sites and has a partial deletion of the E3 region. Approximately 7 days post-transfection, cells and media are harvested and a lysate prepared by repeated freeze-thaw cycles. This 15 lysate is diluted and used to infect 60 -mm dishes of confluent 293 cells for 1 hour. The cells are then overlaid with 0.8% agar/1X MEM/2% calf serum/12.5 mM MgCl₂. Ten days post-infection, individual plaques are to be picked and used to infect 60-mm dishes of 293 cells to 20 expand the amount of virus. Positive plaques are selected for further purification and the generation of adenoviral stocks.

To purify recombinant adenovirus, 150-mm dishes of 75-90% confluent 293 cells are infected with 2-5 PFU/cell, a titer that avoids the potential cytotoxic effects of adenovirus. Thirty hours post-infection, the cells are rinsed, removed from the dishes, pelleted, and resuspended in 10 mM Tris-HCl, pH 8.1. A viral lysate is generated by three freeze-thaw cycles, cell debris is removed by centrifugation for 10 min. at 2,000 rpm, and the adenovirus is purified by density gradient centrifugation. The adenovirus band is stored at -20°C in sterile glycerol/BSA until needed.

The solution of virus particles was sterilized and

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incubated with the implant material (from 6 min to overnight), and the virus-impregnated material was implanted into the osteotomy gap; where viral infection of cells clearly occurred. The results obtained clearly demonstrated the exquisite specificity of the anti- β -gal antibody (Sambrook et al., 1989), and conclusively demonstrated expression of the marker gene product in chondrocyte-like cells of the osteotomy gap. The nuclear-targeted signal has also been observed in pre-osteoblasts.

EXAMPLE X

TRANSFER OF AN OSTEOTROPIC GENE STIMULATES BONE REGENERATION/REPAIR IN VIVO

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In order for a parathyroid hormone (PTH) transgene to function as an osteotropic agent, it is likely that there is a requirement for the PTH/PTHrP receptor to be expressed in the bone repair tissue itself. Therefore, the inventors investigated PTH/PTHrP receptor expression in the rat osteotomy model.

A Northern analysis of poly-A(*) RNA was conducted which demonstrated that the PTH/PHTrP receptor was expression in osteotomy repair tissue (FIG. 13).

The inventors next investigated whether gene transfer could be employed to create transfected cells that constitutively express recombinant hPTH1-34 in vivo, and whether this transgene can stimulate bone formation. The rate of new bone formation is analyzed as follows. At necropsy the osteotomy site is carefully dissected for histomorphometric analysis. The A-P and M-L dimensions of the callus tissue are measured using calipers. Specimens are then immersion fixed in Bouins fixative, washed in ethanol, and demineralized in buffered formic acid. Plastic embedding of decalcified materials is used because of the superior dimensional stability of

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methacrylate during sample preparation and sectioning.

Tissue blocks are dehydrated in increasing alcohol concentrations and embedded. 5 mm thick sections are cut in the coronal plane using a Reichert Polycut microtome. Sections are prepared from midway through the width of the marrow cavity to guard against a sampling bias. Sections for light microscopy are stained using a modified Goldner's trichrome stain, to differentiate bone, osteoid, cartilage, and fibrous tissue. 10 Sections are cover-slipped using Eukitt's mounting medium (Calibrated Instruments, Ardsley, NY). Histomorphometric analyses are performed under brightfield using a Nikon Optiphot Research microscope. Standard point count stereology techniques using a 10 mm x 10 mm eyepiece grid 15 reticular are used.

Total callus area is measured at 125% magnification as an index of the overall intensity of the healing reaction. Area fractions of bone, cartilage, and fibrous tissue are measured at 250 % magnification to examine the relative contribution of each tissue to callus formation. Since the dimensions of the osteotomy gap reflect the baseline (time 0), a measurement of bone area at subsequent time intervals is used to indicate the rate of bone infill. Statistical significance is assessed using analysis of variance, with post-hoc comparisons between groups conducted using Tukey's studentized range t test.

In the 5-mm rat osteotomy model described above, it was found that PTH transgene expression can stimulate bone regeneration/repair in live animals (FIG. 6A, FIG. 6B, FIG. 6C, and FIG. 6D). This is a particularly important finding as it is known that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously, and it is the continuous-type delivery that results from the gene transfer methods used

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here.

Although the present inventors have already demonstrated success of direct gene transfer into regenerating bone in vivo, the use of ex vivo treatment protocols is also contemplated. In such embodiments, bone progenitor cells would be isolated from a particular animal or human subject and maintained in an in vitro environment. Suitable areas of the body from which to 10 obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site) and from the bone marrow. Isolated cells would then be contacted with the DNA (or recombinant viral) composition, with, or preferably without, a matrix, when 15 the cells would take up the DNA (or be infected by the recombinant virus). The stimulated cells would then be returned to the site in the animal or patient where bone repair is to be stimulated.

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EXAMPLE XI

TRANSFER OF GENES TO ACHILLES' TENDON AND TO CRUCIATE LIGAMENT IN VIVO

The studies on regenerating bone described above complement others by the inventors in which gene transfer was successfully employed to introduce genes into Achilles' tendon (FIG 3A, FIG. 3B, FIG. 3C, FIG. 3D, and FIG. 3E) and cruciate ligament (FIG. 4).

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The Achilles' tendon consist of cells and extracellular matrix organized in a characteristic tissue architecture. Tissue wounding can disrupt this architecture and stimulate a wound healing response. The wounded tendon will regenerate, as opposed to scar, if its connective tissue elements remain approximately intact. Regeneration is advantageous because scar tissue is not optimally designed to support normal mechanical

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function. Segmental defects in tendon due to traumatic injury may be treated with biological or synthetic implants that encourage neo-tendon formation. This strategy is limited, however, by the availability of effective (autologous) biological grafts, the long term stability and compatibility of synthetic prostheses, and the slow rate of incorporation often observed with both types of implants.

The inventors hypothesized that the effectiveness of biological grafts may be enhanced by the over-expression of molecules that regulate the tissue regeneration response. Toward this end, they developed a model system in which segmental defects in Achilles' tendon are created and a novel biomaterial, is used as a tendon implant/molecular delivery agent. In the present example, the ability to deliver and express marker gene constructs into regenerating tendon tissue is demonstrated.

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Plasmid (pSVβgal, Promega) stock solutions were prepared according to standard protocols (Sambrook et al., 1989). SIS graft material was prepared from a segment of jejunum of adult pigs (Badylak et al., 1989).

25 At harvest, mesenteric tissues were removed, the segment was inverted, and the mucosa and superficial submucosa were removed by a mechanical abrasion technique. After returning the segment to its original orientation, the serosa and muscle layers were rinsed, sterilized by treatment with dilute peracetic acid, and stored at 4°C until use.

Mongrel dogs (all studies) were anesthetized, intubated, placed in right-lateral recumbency upon a heating pad, and maintained with inhalant anesthesia. A lateral incision from the musculotendinous junction to the plantar fascia was used to expose the Achilles'

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tendon. A double thickness sheet of SIS was wrapped around a central portion of the tendon, both ends were sutured, a 1.5 cm segment of the tendon was removed through a lateral opening in the graft material, and the graft and surgical site were closed. The leg was immobilized for 6 weeks and then used freely for 6 weeks. Graft tissues were harvested at time points indicated below, fixed in Bouins solution, and embedded in paraffin. Tissue sections (8 μ m) were cut and used for immunohistochemistry.

In an initial study, SIS material alone (SIS-alone graft) engrafted and promoted the regeneration of Achilles' tendon following the creation of a segmental defect in mongrel dogs as long as 6 months post surgery. The remodeling process involved the rapid formation of granulation tissue and eventual degradation of the graft. Scar tissue did not form, and evidence of immune-mediated rejection was not observed.

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In a second study, SIS was soaked in a plasmid DNA solution (SIS+plasmid graft) and subsequently implanted as an Achilles' tendon graft (n=2 dogs) or a cruciate ligament graft (n=2 dogs) in normal mongrel dogs. A pSV β gal plasmid that employs simian virus 40 regulatory sequences to drive β -galactosidase (β -gal) activity was detectable by immunohistochemistry using a specific antibody in 4/4 animals. As a negative control, β -gal activity was not detected in the unoperated Achilles' tendon and cruciate ligament of these animals. It appeared, therefore, that SIS facilitated the uptake and subsequent expression of plasmid DNA by wound healing cells in both tendon and ligament.

35 A third study was designed to evaluate the time course of β -gal transgene expression. SIS + plasmid grafts were implanted for 3, 6, 9, and 12 weeks (n=2 dogs

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pr time point) and transgene expression was assayed by
immunohistochemistry and by in situ hybridization.
Cross-sections (8-μm) of Bouins fixed, paraffin embedded
tissue were cut and mounted on ProbeOn Plus slides
(Fisher). Immunohistochemistry was performed according
to the protocol provided with the Histostain-SP kit
(Zymed). In brief, slides were incubated with a well
characterized anti-β-galactosidase antibody (1:200
dilution, 5'→3'), washed in PBS, incubated with a
biotinylated second antibody, washed, stained with the
enzyme conjugate plus a substrate-chromogen mixture, and
then counterstained with hematoxylin and eosin.

Bacterial β-gal activity was detected in tendons
that received the SIS+plasmid graft (8/8 animals).
Although not rigorously quantitative, transgene
expression appeared to peak at 9-12 weeks. Bacterial
β-gal gene expression was not detected in animals that
received SIS-alone grafts (N=2, 3 weeks and 12 weeks).
Again, scar tissue did not form and evidence of immunemediated rejection was not observed.

This study demonstrated that the mucosal biomaterial SIS can function as an autologous graft that promotes the regeneration of tissues such as Achilles' tendon and anterior cruciate ligament. SIS can also be used to deliver a marker gene construct to regenerating tissue.

EXAMPLE XIII

MECHANICAL PROPERTIES OF NEW BONE FORMATION

The mechanical properties of new bone formed during gene transfer may be measured using, e.g., whole bone torsion tests which create a stress state in which the maximum tensile stresses will occur on planes that lie obliquely to the bone's longitudinal axis. Such tests may provide important inferences about the mechanical

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anisotropy of callus tissue and the degree of osseous integration of new bone tissue. These tests are particularly advantageous in the evaluation of fracture specimens, e.g., the irregular shape of callus tissue typically precludes the use of whole bone 4-point bending tests because it is impossible to reproducibly align the points from specimen to specimen.

Femurs are tested on an MTS Servohydraulic Testing Machine while moist and at room temperature. A torque 10 sensor and rotary variable displacement transduces provides data for torque-angular displacement curves. Specially designed fixtures support each bone near the metaphyseal-diaphyseal junctions, and apply a 2-point 15 load to the diaphysis. Tests are conducted at a constant rate of displacement equal to 20 degrees/sec. A 250 inch-ounce load cell measures the total applied force. All bones are tested while moist and room temperature. Torque and angular displacement data are acquired using an analog-to-digital converter and a Macintosh computer 20 and software. From this data, the following variables are calculated: a) maximum torque, b) torsional stiffness, the slope of the pre-yield portion of the curve determined from a linear regression of the data, c) 25 energy to failure, the area under the torque-angular displacement curve to the point of failure, and d) the angular displacement ratio, the ratio of displacement at failure to displacement at yield. Statistical significance is determined Analysis of Variance followed by multiple comparisons with appropriate corrections 30 (e.g., Bonferroni).

This invention also provides a means of using osteotropic gene transfer in connection with reconstructive surgery and various bone remodelling procedures. The techniques described herein may thus be employed in connection with the technology described by

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Yasko et al., 1992; Chen et al., 1991; and Beck et al., 1991, each incorporated herein by reference.

EXAMPLE XIV

TYPE II COLLAGEN PROMOTES NEW BONE GROWTH

Certain matrix materials are capable of stimulating at least some new growth in their own right, i.e., are "osteoconductive materials". Potential examples of such materials are well known in the field of orthopedic research and include preparations of hydroxyapatite; preparations of crushed bone and mineralized collagen; PLGA block copolymers and polyanhydride. The ability of these materials to stimulate new bone formation distinguishes them from inert implant materials such as methylcellulose, which have in the past been used to deliver BMPs to sites of fracture repair.

This Example relates to a study using the rat

20 osteotomy model with implants made of collagen type I

(Sigma), collagen type II (Sigma), and UltraFiber™

(Norian Corp.). These materials have been placed in situ

without DNA of any type. Five animals received an

osteotomy with 10 mg of a type II collagen implant alone

25 (10 mg refers to the original quantity of lyophilized

collagen). Five of five control animals received an

osteotomy with 10 mg of a type I collagen implant alone.

Animals were housed for three weeks after surgery and

then sacrificed.

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The results of these studies were that SIS appeared to retard new bone formation; type I collagen incited a moderately intense inflammatory response; and UltraFiber^m acted as an osteoconductive agent. The type II collagen implant studies yielded surprising results in that 10 mg of this collagen was found to promote new bone formation in the 5-mm osteotomy model (FIG. 22A, FIG. 22B, and FIG.

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22C). New bone - bridging the osteotomy gap - was identified three weeks after surgery in 5/5 animals that received a type II collagen implant alone (i.e., minus DNA of any type). In contrast, fibrous granulation tissue, but no evidence of new bone formation, was obtained in 5/5 animals receiving a type I collagen implant alone.

Radiographic analysis demonstrated conclusively that

all animals receiving an osteotomy with a type II
collagen implant without exception showed radio-dense
material in the osteotomy gap (FIG. 22A). In sharp
contrast, radiographic analysis of all animals receiving
a type I collagen implant revealed no radio-dense

material forming in the osteotomy gap (FIG. 22B). The
arrow in FIG. 22A point to the new bone growth formed in
the osteotomy gap of type II collagen implanted-animals.
No such new bone growth was observed in the animals
receiving type I collagen implants (FIG. 22B).

FIG. 22C demonstrates the results of the osteotomy with a type II collagen implant. The arrow points to the area of new bone formed in the osteotomy gap. In contrast, only fibrous granulation tissue was identified in the type I collagen gap.

Previous studies have suggested that type II collagen plays only a structural role in the extracellular matrix. The results of the type II collagen implant studies are interesting because they demonstrate a novel and osteoconductive role for type II collagen during endochondral bone repair. To further optimize the osteoconductive potential of type II collagen, a yeast expression vector that encodes for type II collagen (full length α1(II) collagen) will be employed to produce recombinant α1(II) collagen protein.

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EXAMPLE XV

IDENTIFICATION OF FURTHER OSTEOTROPIC GENES:

ISOLATION OF A NOVEL LATENT TGF- β BINDING PROTEIN-LIKE (LTBP-3) GENE

The TGF- β s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting 10 of an amino-terminal propeptide followed by mature $TGF-\beta$, two chains of mascent pro-TGF-eta associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer. Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 15 1992). During biosynthesis the mature $TGF-\beta$ dimer is cleaved from the propeptide dimer. TGF- β latency results in part from the non-covalent association of propeptide and mature TGF- β dimers (Pircher et al., 1984 and 1986; Wakefield et al., 1987; Millan et al., 1992; see also 20 Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF- β dimer are also known as the small latent complex. extracellular space small latent complexes must be 25 dissociated to activate mature TGF- β . The mechanism of activation of the latent complex is thought to be one of the most important steps governing $TGF-\beta$ effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et 30 al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF-β binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell

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types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990). Latent TGF- β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- β , but rather it is linked by a disulfide bond to LAP.

Two LTBPs have been isolated to date. The deduced 10 human LTBP-1 amino acid sequence is comprised of a signal peptide, 16 epidermal growth factor-like repeats with the potential to bind calcium (EGF-CB repeats), 2 copies of a unique motif containing 8 cysteine residues, an RGD cell attachment motif, and an 8 amino acid motif identical to 15 the cell binding domain of the laminin B2 chain (Kanzaki et al., 1990). There is evidence that LTBP-1 binds calcium, which, in turn, induces a structural change that protects LTBP from proteolytic attack (Colosetti et al., 1993). LTBP-2 shows 41% sequence identity to LTBP-1, and 20 its structural domains show a similar overall organization (Moren et al., 1994).

While the functions of LTBP-1 and LTBP-2 presently are unknown, several ideas have been put forward in the 25 literature. First, LTBP may regulate the intracellular biosynthesis of latent TGF-eta precursors. Cultured erythroleukemia cells efficiently assemble and secrete large latent TGF-eta complexes, whereas they slowly secrete small latent TGF- β complexes that contain anomalous 30 disulfide bonds (Miyazono et al., 1991; Miyazono et al., 1992). Therefore, LTBP may facilitate the normal assembly and secretion of latent TGF- β complexes. Second, LTBP may target latent TGF- β to specific types of connective tissue. Recent evidence suggests that the 35 large latent $TGF-\beta$ complex is covalently bound to the extracellular matrix via LTBP (Taipale et al., 1994).

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Based on these observations, LTBP has been referred to as a "matrix receptor", i.e. a secreted protein that targets and stores latent growth factors such as $TGF-\beta$ to the extracellular matrix. Third, LTBP may modulate the activation of latent complexes. This idea is based in part on recent evidence which suggests that mature TGF-B is released from extracellular storage sites by proteases such as plasmin and thrombin and that LTBP may protect small latent complexes from proteolytic attack (Falcone 10 et al., 1993; Benezra et al., 1993; Taipale et al., 1994), i.e. protease activity may govern the effect of TGF- β in tissues, but LTBP may modulate this activity. Fourth, LTBP may plays an important role in targeting the latent TGF- β complex to the cell surface, allowing latent TGF- β to be efficiently activated (Flaumenhaft et 15 al., 1993).

A. MATERIALS AND METHODS

20 1. cDNA Cloning

Aliquots (typically 40-50,000 PFU) of phage particles from a cDNA library in the λZAPII® vector made from NIH 3T3 cell mRNA (Stratagene) and fresh overnight XL1-Blue™ cells (grown in Luria broth supplemented with 0.4% maltose in 10 mM MgSO4) were mixed, incubated for 15 min. at 37°C, mixed again with 9 ml of liquid (50°C) top layer agarose (NZY broth plus 0.75% agarose), and then spread evenly onto freshly poured 150 mm NZY-agar plates. 30 Standard methods were used for the preparation of plaque-lifts and filter hybridization (42°C, in buffer containing 50% formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, 100 mg/ml salmon sperm DNA, 100 mg/ml heparin). Filters were washed progressively to high stringency (0.1% SSC/0.1% SDS, 65°C). cDNA probes were radiolabeled 35 by the nick translation method using commercially available reagents and protocols (Nick Translation Kit,

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Boehringer Mannheim). Purified phage clones were converted to pBluescript[®] plasmid clones, which were sequenced using Sequenase (v2.0) as described (Chen et al., 1993; Yin et al., 1995). Sequence alignment and identity was determined using sequence analysis programs from the Genetics Computer Group (MacVector).

2. Tissue In Situ Hybridization

10 To prepare normal sense and antisense probes, a unique 342 bp fragment from the 3' untranslated region (+3973 to +4314, counting the "A" of the initiator Met codon as +1; see "ish", Fig. 1) was subcloned into the pBSKS+ plasmid (Stratagene, Inc.). Template DNA was 15 linearized with either EcoRI or BamHI, extracted, and precipitated with ethanol. Sense and antisense transcripts were generated from 1 mg template with T3 and T7 polymerases in the presence of [35S]UTP at >6 mCi/ml (Amersham, >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining in vitro transcription reagents 20 provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 h, DNA templates were removed by a 15 min. digest at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes were hydrolyzed to an average final length of 25 150 bp by incubating in 40 mM NaHCO3, 60 mM Na2CO3, 80 mM DTT for ~40 min. at 60°C. Hydrolysis was terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to .09 M and 0.56% (v/v), respectively, and the probes were then ethanol precipitated, dissolved in 0.1 M 30 DTT, counted, and stored at -20°C until use. Day

8.5-9.0, day 13.5, and day 16.5 mouse embryo tissue

sections (Novagen) and the in situ hybridization protocol

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were exactly as described (Chen et al., 1993; Yin et al., 1995).

3. Northern Analysis

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MC3T3-E1 cell poly(A+) RNA (2-10 mg aliquots) was electrophoresed on a 1.25% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham). The RNA was cross-linked to the membrane by exposure to a UV light source (1.2 x 10° mJ/cm², UV Stratalinker 2400, Stratagene) and then pre-hybridized for >15 min. at 65°C in Rapid-Hyb buffer (Amersham, Inc.). A specific cDNA probe consisting solely of untranslated sequence from the 3' end of the transcript was ³²P-labeled by random priming and used for hybridization (2 h at 65°C). Blots were washed progressively to high stringency (0.1% SSC/0.1% SDS, 65°C), and then placed against x-ray film with intensifying screens (XAR, Kodak) at -86°C.

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4. Antibody Preparation

LTBP-3 antibodies were raised against a unique peptide sequence found in domain #2 (amino acids 155-167). Peptide #274 (GESVASKHAIYAVC) (SEQ ID NO:16) 25 was synthesized using an ABI model 431A synthesizer employing FastMoc chemistry. The sequence was confirmed using an ABI473 protein sequencer. A cysteine residue was added to the carboxy-terminus to facilitate crosslinking to carrier proteins. For antibody 30 production, the synthetic peptide was coupled to rabbit serum albumin (RSA) using MBS (m-maleimidobenzoic acid-N-hydroxysuccinimide ester) at a substitution of 7.5 mg peptide per mg of RSA. One mg of the peptide-RSA conjugate in 1 ml of Freund's complete adjuvant was 35 injected subcutaneously at 10 different sites along the backs of rabbits. Beginning at 3 weeks after initial

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immunization, the rabbits were given bi-weekly booster injections of 1 mg peptide-RSA in 100 ul of Freund's incomplete adjuvant. IgG was prepared by mixing immune serum with caprylic acid (0.7 ml caprylic acid per ml serum), stirring for 30 min., and centrifuging at 5,000 x g for 10 min. The supernatant was decanted and dialyzed against two changes of phosphate buffered saline (PBS) overnight at 4°C. The antibody solution was then affinity purified by passing it over a column containing the immunizing peptide coupled to Affi-gel 10 affinity support. Bound antibodies were eluted with 0.2 M glycine (pH 2.3), immediately dialyzed against PBS, and concentrated to 1 mg/ml. prior to storage at -70°C.

15 5. Transfection

Transient transfection was performed using standard protocols (Sambrook et al., 1989). Briefly, subconfluent cells (covering -20% of a 100 mm plastic tissue culture dish) were washed 2x in DMEM tissue culture medium (GIBCO) and then incubated for 3 hrs. at 37°C in a sterile mixture of DEAE-dextran (0.25 mg/ml), chloroquine (55 mg/ml), and 15 mg plasmid DNA (Courey and Tjian, 1988). Cells then were shocked by incubation with 10% DMSO in sterile PBS for 2 min. at 37°C, washed 2x with DMEM (Sambrook et al., 1989), and incubated in DMEM plus 10% fetal calf serum and antibiotics for 72 hr. at 37°C.

6. Immunoprecipitation

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For immunoprecipitation, 1 ml of antibody (1:400 final concentration, in PBS-TDS buffer: 0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1% Triton X-100, 0.5% Deoxycholic acid, and 0.1% SDS) was added to 1 ml of radiolabeled medium proteins. The mixture was incubated with shaking at 4°C for 1 hr., protein A-sepharose CL-4B beads were added (200 ml, 10% suspension), and this

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mixture was incubated with shaking for one additional hour at 4°C. Immunoprecipitated proteins were pelleted by brief centrifugation, the pellet was washed 6x with PBS-TDS buffer, 2x protein loading dye was added, and the samples were boiled for 5 min. and then fractionated on 4-18% gradient SDS-PAGE (Bonadio et al., 1985). Cold molecular weight markers (200 kDa-14.3 kDa, Rainbow mix, Amersham) were used to estimate molecular weight. The gel was dried and exposed to film for the indicated time at room temperature.

7. Western Analysis

Fractionated proteins within SDS-polyacrylamide gels

were transferred to a nitrocellulose filter for 2 hours
using Tris-glycine-methanol buffer, pH 8.3 at 0.5 mA/cm².
The filter was blocked, incubated with nonfat milk plus
antibody (1:1000 dilution) for 2 hr, and washed.
Antibody staining was visualized using the ECL Western

blotting reagent (Amersham) according to the
manufacturer's protocols.

B. RESULTS

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25 In this study, the inventors isolated and characterized a novel murine fibrillin-like cDNA encoding LTBP-3. To clone the murine LTBP-3 gene, cDNA from a 3T3 cell cDNA library was amplified using human fibrillin-1 PCR™ primers under low stringency conditions (i.e., 30 annealing at 37°C initially for 10 cycles, followed by annealing at 60°C for 30 cycles). The results indicated that a murine DNA fragment of unexpectedly low homology (~50%) to human fibrillin-1 was obtained. Molecular cloning of the authentic murine fibrillin-1 transcript was also performed, confirming the human and murine 35 fibrillin-1 coding sequences share >95% sequence identity. The murine fibrillin-1 and PCR™ sequences were

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different, which suggested that the PCR™ product may have been derived from a related, fibrillin-like cDNA. The 3T3 cell cDNA library was screened at high stringency using the murine PCR™ product as the probe in order to test this hypothesis. A cDNA walking strategy eventually yielded seven overlapping cDNA clones (FIG. 14). It provides a unique mRNA of 4,314 nucleotides, with an open reading frame of 3,753 nucleotides (SEQ ID NO:2). The deduced molecule is a unique polypeptide of 1,251 amino acids (SEQ ID NO:3). Excluding the signal peptide (21 amino acids), the novel fibrillin-like molecule consists of five structurally distinct regions (Region 1- Region 5), and although similar to murine fibrillin-1 (FIG. 15A), its domain structure is unique as is evidenced by the schematic representation of LTBP-3 shown in FIG. 15B.

Domain #1 is a 28 amino acid segment with a net basic charge (est. pI, 12.36) that may allow for binding acidic molecules in the extracellular matrix (e.g., acidic proteoglycans). Sequences rich in basic amino 20 acids may also function as endoproteolytic processing signals (Barr, 1991; Steiner et al., 1992), which suggests that the NH2-terminus may be proteolytically processed. Domain #2 extends for of 390 amino acids, consisting of an EGF-like repeat, a 135 amino acid 25 segment that was proline-rich (20.7%) and glycine-rich (11.8%) but not cysteine-rich, a Fibmotif (Pereira et al., 1993), an EGF-CB repeat, and a TGF-bp repeat. Domain #3 is a 113 amino acid segment characterized by its high proline content (21%). Domain #4 extends for 30 678 amino acids and consists of 14 consecutive cysteinerich repeats. Based on structural homologies, 12/14 repeats were epidermal growth factor-calcium binding (EGF-CB) motifs (Handford et al., 1991), whereas 2/14 were transforming growth factor- β -binding protein (TGF-35 bp) motifs (Kanzaki et al., 1990). Finally, domain #5 is a 22 amino acid segment at the carboxy-terminus.

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conceptual amino acid sequence encoded by the open reading frame consisted of 1,251 amino acids (FIG. 15B) with an estimated pI of 5.92, a predicted molecular mass of 134,710 Da, and five potential N-linked glycosylation sites. No RGD sequence was present.

Northern blot analysis of murine embryo RNA using a 3' untranslated region probe identified a transcript band of ~4.6 kb. In this regard, 4,310 nt have been isolated by cDNA cloning, including a 3' untranslated region of 401 nt and a 5' upstream sequence of 156 nt. The apparent discrepancy between the Northern analysis result and the cDNA sequence analysis suggested that the 5' upstream sequence may include ~300 nt of additional upstream sequence. This estimate was consistent with preliminary primer extension mapping studies indicating that the 5' upstream sequence is 400-500 nt in length.

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A total of 19 cysteine-rich repeats were found in 20 domains #2 and #4 of the murine LTBP-like (LTBP-3) Thirteen were EGF-like and 11/13 contained polypeptide. the calcium binding consensus sequence. This consensus was derived from an analysis of 154 EGF-CB repeats in 23 different proteins and from structural analyses of the 25 EGF-CB repeat, both bound and unbound to calcium ion (Selander-Sunnerhagen et al., 1992). Variations on the consensus have been noted previously and one of these, D-L-N/D-E-C1, was identified in the third EGF-like repeat of domain #4. In addition, a potential calcium binding sequence which has not previously been reported (E-T-N/D-30 E-C₁) was identified in the first EGF-like repeat of domain #4. Ten of thirteen EGF-CB repeats also contained a second consensus sequence which represents a recognition sequence for an Asp/Asn hydroxylase that co-35 and post-translationally modifies D/N residues (Stenflo et al., 1987; Gronke et al., 1989).

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Although about one-half the size, the deduced polypeptide was organized like fibrillin-1 in that it consisted of a signal peptide followed by 5 structurally distinct domains, i.e., two domains with numerous EGF-like, EGF-CB and Fib repeats and a third with a proline-rich sequence (Pereira et al., 1993). However, comparison of each of these domains using the GAP and BESTFIT programs (Genetics Computer Group) has revealed a low level of amino acid homology of only 27% over the five structural domains shared by the deduced murine polypeptide and human fibrillin-2. These values are low for a putative fibrillin family member because fibrillin-1 and fibrillin-2 share ~50% identity (Zhang et al., 1994).

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A search of available databases revealed that the deduced murine polypeptide was most similar to the human and rat latent TGF- β binding proteins (Kanzaki et al., 1990; Tsuji et al., 1990). In this regard LTBP was found 20 to be similar to fibrillin in that it could also be divided into five structurally distinct domains (FIG. 15A, FIG. 15B, and FIG. 15C). These include a relatively short domain downstream of the signal peptide with a net basic charge (amino acids 21-33, est. pI, 11.14); a 25 domain consisting of EGF-like, EGF-CB, TGF-bp, and Fib motifs plus a proline-rich and glycine-rich sequence (amino acids 34-407); a proline-rich domain (amino acids 408-545); a large, domain consisting of EGF-CB, TGF-bp, and TGF-bp-like repeat motifs (amino acids 546-1379); and a relatively short domain at the carboxy terminus (amino 30 acids 1380-1394). Amino acid sequence comparison of the deduced murine and human polypeptides shows 60% identity for domain #1, 52% identity for domain #2, 30% identity for domain #3, 43% identity for domain #4, and 7% 35 identity for domain #5. The average identity over the five domains shared by the murine polypeptide and human

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LTBP was 38.4%. Significantly, cysteine residues in both polypeptide sequences were highly conserved.

The fibrillins are exclusively expressed by

connective cells in developing tissues (Zhang et al.,
1994), whereas LTBP should be expressed along with TGF-\$\beta\$
by both epithelial and connective cells (Tsuji et al.,
1990). The structural homology data therefore predict
that the murine LTBP-3 gene shown in FIG. 15B should be
expressed by both epithelial and connective tissue cells.
Tissue in situ hybridization was used to test this
hypothesis.

An overview of the expression pattern as determined by tissue in situ hybridization is presented in FIG. 17A, 15 FIG. 17B, FIG. 17C, and FIG. 17D. Approximate midsagittal sections of normal murine embryos at days 8.5-9.0, 13.5 and 16.5 p.c. of development were hybridized with a 35S-labeled single stranded normal sense riboprobe from the same cDNA construct was used. At day 8.5-9.0 of 20 development, intense gene expression was observed in the mesometrial and anti-mesometrial uterine tissues, ectoplacental cone, placenta, placental membranes. transcript appeared to be widely expressed in murine embryo mesenchymal/connective tissue compartments, 25 including the facial mesenchyme, at days 8.5-9.0, 13.5 and 16.5 of development. Particularly intense expression of the transcript was noted in the liver.

Microscopy of day 8.5-9.0 embryos confirmed the widespread expression of the murine gene by mesenchymal cells. Significant expression of the transcript by cells of the developing central nervous system, somites and

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cardiovascular tissue (myocardium plus endocardium) was also observed.

Microscopy of day 13.5 and day 16.5 embryos demonstrated expression of the murine gene by skeletal muscle cells and by cells involved in intramembranous and endochondral bone formation. The transcript was expressed by osteoblasts and by periosteal cells of the calvarium, mandible and maxilla. The transcript was also identified in both cartilage and bone of the lower extremity. A positive signal was detected in perichondrial cells and chondrocytes (proliferating > mature > hypertrophic) of articular cartilage, the presumptive growth plate, and the cartilage model within the central canal. The positive signal was also expressed by blood vessel endothelial cells within the mid-diaphysis, and the surrounding muscle cells (FIG. 18A, FIG. 18B, FIG. 18C, FIG. 18D, FIG. 18E, FIG. 18F, FIG. 19G, FIG. 18H, FIG. 18I, FIG. 18J, FIG. 18K, FIG. 18L, FIG. 18M, FIG. 18N, FIG. 18O, and FIG. 18P).

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Respiratory epithelial cells lining developing small airways and connective tissue cells in the pulmonary interstitium expressed the murine transcript, as did myocardial cells (atria and ventricles) and endocardial 25 cushion tissue. Cells within the walls of large arteries also expressed the transcript. Expression of the murine gene was identified in several organs of the alimentary system, including the tongue, esophagus, stomach, small and large intestine, pancreas and liver. Mucosal 30 epithelial cells lining the upper and lower digestive tract plus the smooth muscle and connective tissue cells found in the submucosa expressed the transcript, as did acinar cells of the exocrine pancreas. Despite the high level of transcript expression in the liver, these 35 results suggest both cell populations express the LTBP-3 transcript.

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In the kidney, expression above the basal level was observed in cells of developing nephrons, the ureteric bud, kidney blastema and the kidney interstitium. In the skin, epidermal and adnexal keratinocytes, dermal connective tissue cells, and brown fat cells within the dorsal subcutis expressed the murine transcript. In the central and peripheral nervous systems, ganglion cells within the cerebrum, brainstem, spinal cord, and peripheral nerves expressed the murine transcript. The transcript was also intensely expressed by cells of the developing murine retina.

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Thus, the murine gene is widely expressed by both epithelial and connective tissue cells, a pattern that would be expected for a latent TGF- β binding protein. 15 Three final observations argue that the LTBP-like (LTBP-3) sequence presented in FIG. 25 is not simply the murine homologue of human LTBP. First, domain #4 of the murine LTBP-like (LTBP-3) sequence has a smaller number of EGFlike repeat motifs than human and rat LTBP (8 versus 11). 20 Second, portions of the human and rat LTBP-like coding sequence were characterized and found to share ~90% identity with human and rat LTBP but only 65% identity with the murine LTBP-like gene. Third, the human LTBP and LTBP-like genes are localized to separate 25 chromosomes. Human LTBP was assigned to human chromosome 2 based on the analysis of human x rodent somatic cell hybrid lines (Stenman et al., 1994). The present invention represents the first mapping of an LTBP gene in 30 the murine. The human LTBP-like genes was recently localized to chromosome 11 band q12, while the murine gene was mapped to murine chromosome 19, band B (a region of conserved synteny), using several independent approaches, including fluorescent in situ hybridization. 35

The first indication of alternative splicing came from molecular cloning studies in the murine, in which

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independent cDNA clones were isolated with a deletion of 51 bp from the coding sequence. PCR^m/Southern blot analysis provided additional evidence that the homologous 51 bp sequence was alternatively spliced in normal murine embryo tissues.

Northern blot analysis also demonstrated that the novel fibrillin gene was also expressed in rat callus three weeks after osteotomy, after mineralization has begun. Gene expression in normal adult rat bone tissue was insignificant, which suggests that microfibrils are an important part of the bone fracture healing response. The novel fibrillin-like gene was expressed in callus as a pair of alternatively spliced transcripts. This result has been independently reproduced on three occasions. Molecular cloning of the novel fibrillin gene in both murine and rat has identified potential splice junction sites for the alternative splicing event.

MC3T3-El murine pre-osteoblasts were used to demonstrate that the murine gene product was capable of binding TGF-β. MC3T3-El cells were utilized because they synthesize and secrete TGF-β, which may act as an autocrine regulator of osteoblast proliferation (Amarnani et al., 1993; Van Vlasselaer et al., 1994; Lopez-Casillas et al., 1994).

To determine whether or not MC3T3-E1 cells co-expressed the murine gene product of TGF-β, cells were plated on 100-mm dishes under differentiating conditions (Quarles et al., 1992) and the medium was replaced twice weekly. Parallel dishes were plated and assayed for cell number and alkaline phosphatase activity, which confirmed that osteoblast differentiation was indeed taking place. Equal aliquots of total cellular RNA was prepared from these MC3T3-E1 cells after 5, 14 and 28 days in culture for Northern blot analysis. As shown in FIG. 19,

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expression of the new murine gene peaked on day 14 of culture. Since MC3T3-E1 cells also show a peak in alkaline phosphatase activity on day 14 of culture (Quarles et al., 1992), the results suggest for the first time that LTBP-2 gene expression is an early marker of osteoblast differentiation.

C. DISCUSSION

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10 This study reports the molecular cloning of a novel LTBP-like gene that contains numerous EGF-like repeats. Northern analysis indicates that the gene encodes a single transcript of -4.6 kb in murine embryo tissues. The deduced amino acid sequence of the murine gene product appears to be a secreted polypeptide of 1,251 15 amino acids. Although it is similar to fibrillin, the overall structural organization and expression pattern of this gene product most resembles LTBP, a latent TGF- β binding protein that was originally isolated and characterized by Heldin and co-workers (Kanzaki et al., 20 1990). Several observations strongly suggest that LTBP and the murine LTBP-like gene product are therefore derived from related but distinct genetic loci. First, LTBP and the LTBP-like coding sequence share -40% identity and differences exist in the number of EGF-CB 25 repeats in the deduced polypeptide sequence of the two molecules. Second, a portion of the murine LTBP gene has been cloned and shown to share ~90% identity with human and rat LTBP. Conversely, portions of the human and rat LTBP-like genes have been cloned and shown to share -90% 30 identity with the murine LTBP-like gene. Third, LTBP and the LTBP-like gene reside on different human chromosomes (Stenman et al., 1994). Taken together, these data suggest that a family of at least two LTBP genes exists.

Similarities in the structural organization of LTBP-1 and the fibrillin-1 and fibrillin-2 polypeptides have

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been noted previously (Pereira et al., 1993; Zhang et al., 1994; Taipale et al., 1994). For example, LTBP-1 and the fibrillins are all secreted extracellular matrix constituents. Moreover, each polypeptide can be organized into five domains, two of which consists predominantly of EGF-CB and TGF-bp repeat motifs. LTBP-1 and fibrillin-1 also share a domain that is proline-rich, and LTBP possesses an 8-cysteine repeat previously referred to as the "Fib motif" because it was assumed to 10 be unique to fibrillin (Pereira et al., 1993). similarities likely explain the initial isolation and cloning of the LTBP-2 PCR™ product, especially since the human oligonucleotide primers used to initially amplify murine cDNA were designed to direct the synthesis of an EGF-CB repeat in domain #4. 15

Another point of distinction between LTBP-2 and fibrillin concerns the spacing of conserved cysteines C4 and C5 in EGF-like repeats. Fibrillin-1 and fibrillin-2 each contain >50 such repeats, and in every one the 20 spacing is C_4 -X- C_5 . While this pattern is repeated in a majority of the EGF-like repeats in LTBP-1 and LTBP-2, both genes also contain repeats with the spacing $C_4-X-X C_5$. Although the significance of this observation is unclear, variation in the number of amino acids between C_4 25 and C_{S} would not be expected to alter the function of the EGF-like repeat. Mature EGF is a 48 amino acid secreted polypeptide consisting of two subdomains that have few interdomain contacts (Engel, 1989; Davis, 1990). larger NH2-terminal subdomain consists of residues 1-32 30 and is stabilized by a pair of disulfide bonds (C1-C3 and C_2 - C_4), whereas the smaller COOH-terminal subdomain (amino acids 33-48) is stabilized by a single disulfide bond (C_5 - C_6). The COOH-terminal subdomain has a highly conserved conformation that only is possible if certain residues 35 and the distances between them are well conserved, while conformation-sequence requirements for the NH2-terminal

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subdomain are relatively relaxed. Variation in C_4 - C_5 spacing would not be expected to alter conformation because these residues do not normally form a disulfide bond and the spacing variation occurs at the interface of subdomains that would not be predicted to interact. The cloning of additional genes will decide whether variation in C_4 - C_5 spacing is a reliable discriminator between members of the LTBP and fibrillin gene families.

10 The LTBP-2 gene is expressed more widely during development than fibrillin-1 or fibrillin-2. Studies in developing murine tissues have shown that the Fbn-1 gene is expressed by mesenchymal cells of developing connective tissue, whereas the murine LTBP-like gene is 15 intensely expressed by epithelial, parenchymal and stromal cells. Earlier reports have suggested that $TGF-\beta$ plays a role in differentiation and morphogenesis during murine development (Lyons and Moses, 1990), when TGF- β is produced by epithelial, parenchymal and stromal cells. Tsuji et al., (1990) and others have suggested that the 20 expression of TGF- β binding proteins should mirror that of TGF- β itself; the expression pattern of the LTBP-2 gene over the course of murine development is consistent with this expectation. However, the LTBP-2 gene may not be completely co-regulated with TGF- β . TGF- β gene and 25 protein expression during murine development has been surveyed extensively (Heine et al., 1987; Lehnert and Akhurst, 1988; Pelton et al., 1989; Pelton et al., 1990a,b; Millan et al., 1991); these studies have not identified expression by skeletal muscle cells, 30 chondrocytes, hepatocytes, ganglion cells, mucosal cells lining the gut, and epithelial cells of developing nephrons. It is conceivable that the LTBP-2 molecule has

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an additional function in certain connective tissues besides targeting $TGF-\beta$.

The binding properties of the LTBP-2 gene product

are under investigation. Formally, the LTBP-2

polypeptide may bind a specific TGF-β isoform, another

member of the TGF-β superfamily (e.g., a bone

morphogenetic protein, inhibin, activin, or Mullerian

inhibiting factor), or a growth factor unrelated to TGF
β. Anti-peptide antibodies to the murine LTBP-2

polypeptide have been generated and osteoblast cell lines

that express the molecule at relatively high levels have

been identified. Studies with these reagents suggest

that LTBP-2 assembles intracellularly into large latent

complexes with a growth factor that is being

characterized by immunological methods.

The presence of dibasic amino acids in the LTBP-2 sequence suggests that it may undergo cell- and tissue-20 specific proteolysis. TGF- β regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase 25 inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent review, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; Miyazono et al., 1992). Conversely, 30 production of extracellular matrix has been shown to down regulate TGF- β gene expression (Streuli et al., 1993). TGF- β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of 35 genes. LTBP-1 and LTBP-2 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor complexes and then targeting

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the complex to specific connective tissues (Taipale et al., 1994).

If LTBP-3 is like LTBP-1, it has the potential to function as a secreted, extracellular structural protein. As demonstrated here, domain #1 of LTBP-3 appears to be a unique sequence that likely has a globular conformation. Domain #1 also is highly basic and may facilitate LTBP-2 binding to acidic molecules (e.g., acidic proteoglycans) within the extracellular space. Sequences rich in basic 10 amino acids have also been shown to function as endoproteolytic processing signals for several peptide hormones (Barr, 1991; Steiner et al., 1992). possible, therefore, that the NH2-terminus of LTBP-3 is 15 proteolytically processed in a tissue-specific manner. Domains #2 and #4 consist of consecutive cysteine-rich repeats, the majority of which are of the EGF-CB type. Besides binding calcium (Corson et al., 1993), these repeats may provide LTBP-3 with regions conformation 20 capable of interacting with other matrix macromolecules (Engel, 1989). Domain #3 is proline rich and may be capable of bending (or functioning like a hinge) in three-dimensional space (MacArthur and Thornton, 1991). (In this regard, domain #2 is of interest because it has 25 a similar stretch of 135 amino acids that is both proline- and glycine-rich. Since glycine-rich sequences are also thought to be capable of bending or functioning like a hinge in three-dimensional space, this amino acid sequence may interrupt the extended conformation of domain #2, thereby providing it with a certain degree of 30 flexibility in three-dimensional space.) Domain #5 also appears to be a unique sequence having a globular conformation. The absence of a known cell attachment motif may indicate that, in contrast to LTBP-1, the LTBP-3 molecule may have a more limited role in the 35 extracellular matrix (i.e., that of a structural protein)

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in addition to its ability to target latent $TGF-\beta$ complexes to specific connective tissues.

MC3T3-E1 pre-osteoblasts co-express LTBP-3 and TGF- β l and these proteins form a complex in the culture 5 These results are particularly interesting because bone represents one of the largest known repositories of latent TGF- β (200 μ g/kg bone; Seyedin et al., 1986 and 1987), and because this growth factor plays a critical role in the determination of bone 10 structure and function. For example, TGF-eta is thought to (i) provide a powerful stimulus to bone formation in developing tissues, (ii) function as a possible *coupling factor" during bone remodeling (a process that coordinates bone resorption and formation), and (iii) 15 exert a powerful bone inductive stimulus following fracture. Activation of the latent complex may be an important step governing TGF-eta effects, and LTBP may modulate the activation process (e.g., it may "protect" small latent complexes from proteolytic attack). 20

Expression of large latent TGF- β complexes bearing LTBP may be physiologically relevant to, i.e., may be part of the mechanism of, the pre-osteoblast → osteoblast differentiation cascade. This is based on the evidence 25 that MC3T3-E1 cells express large latent TGF- β 1 complexes bearing LTBP-2 precisely at the time of transition from the pre-osteoblast to osteoblast phenotype (~day 14 in culture, or, at the onset of alkaline phosphatase expression; see Quarles et al., 1992). The organ culture 30 model, for example, likely is comprised of differentiated osteoblasts but few bond progenitors, making it a difficult model at best in which to study the differentiation cascade (Dallas et al., 1984). also well known that MG63, ROS17/2.8 and UMR 106 cells are rapidly dividing and they express the osteoblast phenotype. Thus, these osteoblast-like cell lines do not

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show the uncoupling of cell proliferation and cell differentiation that characterizes the normal (physiologically relevant) pre-osteoblast \rightarrow osteoblast transition (Gerstenfeld et al., 1984; Stein and Lian, 1993). Therefore, the production of small versus large latent TGF- β complexes may be associated with specific stages in the maturation of bone cells.

LTBP-3 may bind calcium, since EGF-CB repeats have been shown to mediate high affinity calcium binding in 10 LTBP-1 and other proteins (Colosetti et al., 1993). Calcium binding, in turn, may contribute to molecular conformation and the regulation of its interactions with other molecules. The presence of dibasic amino acids suggests that LTBP-3 may also undergo cell- and 15 tissue-specific proteolysis. TGF- β regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase; plasminogen activator, and 20 stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent reviews, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; and Miyanzono et al., 25 1993). Conversely, production of extracellular matrix has been shown to down regulate $TGF-\beta$ gene expression (Streuli et al., 1993). TGF- β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a 30 relatively large number of genes. LTBP-1, LTBP-2, and LTBP-3 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor

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complexes and then targeting the complex to specific connective tissues (Taipale et al., 1994).

EXAMPLE XVI

5 PREPARATION OF ANTIBODIES AGAINST THE LTBP-3 GENE PRODUCT

An affinity-purified antibody (#274) capable of immunoprecipitating was prepared against the murine LTBP-3 gene product. A full-length murine cDNA was assembled into the pcDNA3 mammalian expression vector (Invitrogen) and expressed following transient transfection of 293T cells. Nascent polypeptides, radiolabeled by addition of 35 S Cys to the medium of transfected cells, were immunoprecipitated using affinity-purified antibody #274. As shown in FIG. 20, the new murine polypeptide was estimated to be 180-190 kDa. To ensure the specificity of #274 binding, we showed that preincubation with 10 μ g of synthetic peptide blocks immunoprecipitation of the 180-190 kDa band.

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Finally, MC3T3-E1 cells were cultured for 7 days under differentiating conditions and double-labeled with 30 μ Ci/ml ³⁵S cysteine and ³⁵S methionine in deficient media. Radiolabeled media was dialyzed into cold PBS with protease inhibitors. Aliquots of the dialyzed medium sample (106 incorporated CPM) were analyzed by a combined immunoprecipitation/Western analysis protocol. The murine polypeptide was clearly and reproducibly secreted by MC3T3-E1 cells, migrating under reducing conditions as a single band of 180-190 kDa (FIG. 21). Consistent with the results of previous studies (e.g., Miyazono et al., 1988; Dallas et al., 1994; Moren et al., 1994), bands of 70 and 50 kDa corresponding to the TGF- β 1 precursor were co-immunoprecipitated with the 180 kDa LTBP-3 protein. Weak bands of 40 and 12 kDa were also identified in experiments in which only immunoprecipitation was performed. The latter were not

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included in FIG. 21 because they migrated within that portion of the gel included in the Western analysis. Protein bands of 70-12.5 kDa are not variant forms of LTBP-3; FIG. 20 demonstrates that LTBP-3 migrates as a single band of 180-190 kDa following transient 5 transfection of 293T cells, which fail to make TGF- β 1. By immunoprecipitation, a unique band consistent with monomeric mature TGF- β 1 was found in the LTBP-2 immunoprecipitate. Antibody #274 is incapable of binding TGF- β 1 as determined by radioimmunoassay using 10 commercially available reagents (R&D Systems) and the manufacturer's suggested protocols. These results have been reproduced in 6 independent experiments which utilized 3 separate lots of MC3T3-E1 medium. new murine LTBP-3 polypeptide binds TGF- β in vitro. 15

EXAMPLE XVII ISOLATION OF A GENE ENCODING MURINE LTBP-2

In addition to determining the DNA and corresponding polypeptide sequence of the murine LTBP-3 gene, the murine LTBP-2 gene was also cloned and sequenced.

The complete cDNA nucleotide sequence for murine 25 LTBP-2 is shown in FIG. 27 (SEQ ID NO:17). The deduced amino acid sequence is shown in FIG. 28 (SEQ ID NO:18).

EXAMPLE XVIII

30 EXPRESSION OF RECOMBINANT TYPE II COLLAGEN

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The Pichia Expression Kit (Invitrogen, Inc.) may be used to prepare recombinant type II collagen. This kit, based on the methylotrophic yeast, Pichia pastoris, allows high-level expression of recombinant protein in an easy-to-use relatively inexpensive system. In the absence of the preferred carbon source, glucose, P.

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pastoris utilizes methanol as a carbon source. The AOXI promoter controls the gene that codes for the expression of the enzyme alcohol oxidase, which catalyzes the first step in the metabolism of methanol. This promoter, which is induced by methanol, has been characterized and incorporated into a series of Pichia expression vectors. This feature of Pichia has been exploited to express high levels of recombinant proteins often in the range of grams per liter. Because it is eukaryotic, P. pastoris utilizes posttranslational modification pathways that are similar to those used by mammalian cells. This implies that the recombinant type II collagen will be glycosylated and will contain disulfide bonds.

The inventors contemplate the following particular elements to be useful in the expression of recombinant type II collagen: the DNA sequence of human type II collagen (SEQ ID NO:11) (Lee et al., 1989); rat type II collagen (SEQ ID NO:13) (Michaelson, et al., 1994);

and/or mouse type II collagen (SEQ ID NO:15) (Ortman, et al., 1994). As other sources of DNA sequences encoding type II collagen are available, these three are examples of many sequence elements that may be useful in the present invention.

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For preparation of a recombinant type II collagen, the native type II collagen cDNA is modified by the addition of a commercially available epitope tag (the HA epitope, Pharmacia, LKB Biotechnology, Inc.). Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production. (PCR[™] is a registered trademark of Hoffmann-LaRoche, Inc.). This is followed by cloning into the

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Pichia expression vector. The resulting plasmid is characterized by DNA sequence analysis, linearized by digestion with NotI, and spheroplasts will be prepared and transformed with the linearized construct according to the manufacturer's recommendations.

Transformation facilitates a recombination event in vivo between the 5' and 3' AOX1 sequences in the Pichia vector and those in the Pichia genome. The result is the replacement of AOX1 with the gene of interest.

Transformants are then plated on histidine-deficient media, which will select for successfully transformed cells. Transformants are further selected against slow growth on growth media containing methanol. Positive transformants are grown for 2 days in liquid culture and then for 2-6 days in broth that uses methanol as the sole carbon source. Protein expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western hybridization using a commercially available polyclonal antisera to the HA epitope (Pharmacia).

Recombinant type II collagen protein can be purified according to the manufacturer's recommendations, dialyzed against double distilled, deionized water and lyophilized in 10 mg aliquots. The aliquots are sterilized and used as implant material for the osteoconductive matrices.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

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may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

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		(C) CITY: Ann Arbor
10		(D) STATE: Michigan
		(E) COUNTRY: United States of America
		(F) POSTAL (ZIP) CODE: 48109-1280
	(ii)	INVENTORS: BONADIO, Jeffrey
15		ROESSLER, Blake J.
		GOLDSTEIN, Steven A.
		LIN, Wushan
	(iii)	TITLE OF INVENTION: METHODS AND COMPOSITIONS
20		FOR STIMULATING BONE CELLS
	(iv)	NUMBER OF SEQUENCES: 18
	(v)	CORRESPONDENCE ADDRESS:
25		(A) ADDRESSEE: Arnold, White & Durkee
		(B) STREET: P.O. Box 4433
		(C) CITY: Houston
		(D) STATE: Texas
		(E) COUNTRY: United States of America
30		(F) ZIP: 77210
	(vi)	COMPUTER READABLE FORM:
		(A) MEDIUM TYPE: Floppy disk
		(B) COMPUTER: IBM PC compatible
35		(C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII
		(D) SOFTWARE: PatentIn Release #1.0, Version

#1.30

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(vii)	CURRENT	APPLICATION	DATA:
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- (A) APPLICATION NUMBER: UNKNOWN
- (B) FILING DATE: CONCURRENTLY HEREWITH
 - (C) CLASSIFICATION: UNKNOWN

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(viii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/316,650
- (B) FILING DATE: 30-SEP-1994
- (C) CLASSIFICATION: UNKNOWN

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- (A) APPLICATION NUMBER: US 08/199,780
- (B) FILING DATE: 18-FEB-1994
- (C) CLASSIFICATION: UNKNOWN
- 15 (ix) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Parker, David L.
 - (B) REGISTRATION NUMBER: 32,165
 - (C) REFERENCE/DOCKET NUMBER: UMIC009P--
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 - (A) TELEPHONE: (512) 418-3000
 - (B) TELEFAX: (713) 789-2679
 - (C) TELEX: 79-0924

25

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 417 amino acids
- 30
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

NO:1:
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SEO
DESCRIPTION
SEQUENCE
(xi)

Val	Lys	Gly	Met	Pro 80	
Gln 15	СІУ Lуз	Ser Gly	Gln	Ile.Pro 80	
Сув	Thr 30	Arg		Val	
Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val 1 15	Glu	Ile Gln Gly His Ala Gly Gly Arg Arg 40	Leu Leu	Phe Gly Leu Arg Arg Pro Gln Pro Ser Lys Ser Ala 70	
Leu	Pro	Gly	Thr 60	Ser	
Val	Thr Asp Ala Ser Leu Met 25	$_{ m G1y}$	Glu Ala	Ly в 75	
Val	Leu	Ala	Glu	Ser	
Met	Ser 25	His	Ser His Glu Leu Leu Arg Asp Phe 50 55	Pro	
Leu	Ala	Gly 40	Asp	Gln	
Met	Asp	Gln	Arg 55	Pro	
Arg		Ile	Leu	Arg 70	
A9n 5	Ala	Glu	Leu	Arg	
Gly	Leu Leu Gly Gly Ala 20	Lys Lys Val Ala Glu 35	Glu	Arg	
Pro	Gly	Val 35	His	Leu	
Ile	Leu	Lys		Gly	
Met 1	Leu	ьув	Gln	Phe 65	

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Glu Glu Gln Ser Gln Gly Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala 100

Asp Tyr Met Ser Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu Glu

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Glu	Asn	Arg 160	Phe	Val	His	\rg	Thr
Leu	Phe	Leu			Arg 1	Leu Arg	Val 1
Glu Glu His Leu Glu 125	Phe	Glu Leu Arg 160	Gln	Glu Met 190	/al	Val I	Glu v
Glu 125	Phe	Ala	Glu (Ma (Leu 1 205		ile G
	Arg 140	Ser	Trp Glu Gln Gly 175	Pro 1	Ser Leu Val 205	Pro Ala 220	la I
His His	Phe	Ser 155	Asp '	Pro Pro Ala	Thr 8	Ser F	Leu A
His	Ala	Ile	Pro Asp 170		Asp 7	/al s	ly L
Phe	Ser		Gly	Met Lys 185	Thr Arg Leu Leu Asp 200	Asp Val	ሃ ፑ G
Ser 120	Glu Ser 135	Glu Asn Glu Val 150	Glu Gln Val Asp Gln Gly 165		Leu 1 200	Phe A	T na
Ser	Glu 135	Asn	Азр	Tyr Glu Val	Arg 1		ro A
Val	Ser	Glu 150	Val	Tyr	Thr 1	Trp Glu Thr 215	Gln P 230
Thr	Thr	Pro	Gln 165	ile	[]e	ĝ.	уя G 2
Ala Asn 115	Gly Thr	Ile	Glu	Asn 180	ren 1	krg 1	lu L
	Pro	Ser	Arg	₩e t	His 1	Val Thr Arg 210	rg G
Ser	11e 130	Ser	Phe	Arg Met	31y 1	Val 1 210	hr A
Ser	Asn	Leu 145	Leu	нів	Pro Gly His Leu Ile 195	Asn V	Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala 225
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			10	15		70	

Ser	Leu	Arg	Lys	Val 320	Tyr	Thr	Ile
11e 255	Pro	Arg Arg	Lys	Asp	Phe 335	Ser	Ser
Ser	Arg 270	Thr	Ser	Ser	Ala	Asn 350	
Val	Gln Leu	Leu 285	Ser	Phe	Gln	Leu Asn 350	Asn :
His		Thr	Arg 300	Авр	Tyr	His	/al /
Gly Gln His 250	Ala	нів	Pro Gln Arg	Val 315	Pro Gly Tyr Gln Ala 330	Авр 1	Ser 1
G1y 250	Asn Trp 265	Gly	Pro	Tyr	Pro 330	Ala	Asn S
Gln	Asn 265	Gly His Asp Gly Arg Gly His 280	Pro Lys His His 295	Ser Leu Tyr Val Asp Phe 315	Pro	Pro Leu Ala Asp His 345	Leu Val Asn Ser Val Asn Ser 360
His	Gly	G1Y 280	нів	Ser	Ala	Pro	Leu '
Thr	Ser	Asp	L ув 295	нів	Val	Phe	Thr
Arg	$_{ m G1y}$	His	Pro	Arg 310	Ile	Pro	Gln Thr
Thr 245	Pro Gln Gly 260	Gly	Ser	Arg Arg His 310	Trp 325	Сув	Val (
His Leu His Gln Thr Arg Thr 245	Pro 260	Phe	Arg	Сув	Asn Asp Trp Ile Val 325	Gly Asp Cys 340	Ile
H18	Ser Leu	Thr 275	Ala Lys 290	Asn	Asn	Gly	Ala 355
ren -	Ser	Val	Ala 290	Гув	Trp	His	His
нтв	Arg	Leu	Ser	Asn 305	$_{ m G1}_{ m y}$	Сув	Asn
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Leu		Met	400
Met		Glu	
Ser		Tyr Gln	
Ile		Tyr	
Ala	380	Asn	
Ser	380	Lys	395
Glu Leu		Leu	
Glu	•	Val Leu	
Thr		Val	
Pro	375	Lys	
Val		Asp	390
Сув		Tyr	
сув сув		glu	
Ala		Asp	
Lys	370	Leu	
Pro		Tyr	385

S

Val Val Glu Gly Cys Gly Cys Arg Tyr Pro Tyr Asp Val Pro Asp Tyr 405

10 Ala

(2) INFORMATION FOR SEQ ID NO:2:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3753 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

20

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..3753

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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4 8	96	144	192
		. •	
CTG	CAG Gln	CCT	TGT
GCG Ala	GCA Ala	GCG	AGC Ser
CTG	GGG G1y 30	TTT	GAC ABP
CTC CTG Leu Leu	AGC Ser	GTC Val	TGT CGG GAC Cys Arg Asp
CTC	GGC	rrg /al	TGT
GCA CTA Ala Leu	CCG	AAG	CAG
GCA Ala 10	GTG GGC CGG CCG Val Gly Arg Pro 25	TTC	GGC
CTG	GGC G1Y 25	CGC	AAG Lyb
CTG	GTG Val	CAA Gln 40	CTG
666 61y	GGG	GCC	TGT
TTG	CGA	TGG	ACC
GCA Ala 5	GGC G17 7	CGC	CGG
GCC Ala	GGC G1y G	GGG G1y	AAG CGG Lys Arg
CAG Gln	CCC Pro	GCG Ala 35	TGC Cys
CGC	GGC	GGG	ATC Ile
ATG Met	CTG	GCG	GTG
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240	288	336	384	432	480
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ACC Thr 80	CCC	CCC	GGA Gly	TCC	AGC
AGC		TGT Cys	ACC	ATG	GCT
CAC His	CCT CTA Pro Leu 95	CTG Leu 110	GGA Gly		GTG
GGC Gly	TGC Cys	твс	GCA Ala 125		TCT
GAG AAC GGC Glu Asn Gly 75	GTG Val	CAG Gln	GCT Ala	GAC CGG Asp Arg 140	GAG Glu
GAG Glu 75	TTC CGC GTG GTG Phe Arg Val Val 90	TCT TCC CGA AAC Ser Ser Arg Asn 105	CCT	CCC Pro	CCA GAA GGA GAG Pro Glu Gly Glu
GGA Gly	GTG Val 90	cga Arg	GTG Val	TGG	GAA Glu
CTC ATC Leu Ile	TTC CGC Phe Arg	TCC Ser 105	CAG Gln	GGC	CCA Pro
CIC	TTC	TCT Ser	TGC Cys 120	CCC	GCC
ACG	GCC	TGC	TTC	GGC Gly 135	CTT
ATG Met 70	TCT	CAG Gln	CGC	TCA	CCC
TCC AAC Ser Asn	GGT Gly 85	66C 61y	GGG G1y	GGG AGT Gly Ser	CCG
TCC	ACC	GGT Gly 100	TTC ACG GGG CGC TTC Phe Thr Gly Arg Phe 115	GGG	CTG
GGC	CTC	AAC	TTC Phe	ACC	CCG
CAG Gln	ACG	ATG Met	gat Abp	GGC Gly 130	GGC Gly
CAG Gln 65	GAC	TGC	CCG	GCT	ACA Thr
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528	576	624	672	720	768
TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro 165	CCT CCT GCA CAT GCA GCC TTC TTG GTG CCC CTG GGG Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly 180	ATC TCG GCA GAA GTG CAG GCT CCG CCC CTG GTG AAC Ile Ser Ala Glu Val Gln Ala Pro Pro Pro Val Val Asn 200	CAT CAC CCT CCT GAA GCT TCC GTT CAG GTG CAC CGC ATC His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile 215	CCG AAC GCT GAA GGC CCA GCC TCT TCC CAG CAC TTG CTG CCG 720 Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro 230 230	CCC CCG CAC CCG AGG CCA CCC ACT CAA AAG CCA CTG GGC 768 Pro Pro His Pro Arg Pro Thr Gln Lys Pro Leu Gly
CAC GCC ATT His Ala Ile	GRG GGT Glu Gly	A CAA Y Gln 195	T GTC g Val 0	G CCG	AAG Lys
AAA CA Lys Hi	666 GA 61y G1	CCA GGA Pro Gly	GTG CGT Val Arg 210	GAG GGG Glu Gly 225	CAT CCC His Pro
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CCT	ACT	ACA Thr	TGC Cys 320	AAC	AC Isn
AAC Asn	GGT Gly	TAT Tyr	GAC	ATC 1 Ile 1 335	AC P
AGC Ser 270		CAG Gln	GCT Ala	GAT / Asp]	CTC AAC AAC Leu Asn Asn 350
66C 61y	GGT AGC ATC Gly Ser Ile 285	CTT	GAG GTG GGT GCT GAC Glu Val Gly Ala Asp 315	CAG Gln A	TGC C Cys L
TGT	GGT	CAG Gln 300	GTG (TGC CAG Cys Gln	AC T
OCT. Pro	TGC	CCA	CCT GTA CCT GTA CGT GGG GAG GTG GGT Pro Val Pro Val Arg Gly Glu Val Gly 310	CAC :	GGT GAC TGC Gly Asp Cys
CCC AAG CAG Pro Lys Gln 265	CAG GAA GAT TGC Gln Glu Asp Cys 280	ТСС	666 61y	ACC Thr 1330	
AAG Lys 265	gat Asp	AAG Lyb	CGT	AGG CTC AAC AGC ACC Arg Leu Asn Ser Thr 330	TGC CAT Cys His
CCC	GAA Glu 280	CAC	CCT GTA CGT Pro Val Arg	AAC A	3TG 1 /al (
TTG	CAG Gln	TGT Cys 295	CCT	CTC AAC Leu Asn	AAT C
ACA	AAG	аад Гув	GTA Val 310	AGG	3GG 1
TGC TTC CAG GAC ACA Cys Phe Gln Asp Thr 260	ACC		CCT	AAG Lys 325	GCG ATG CCC GGG AAT GTG Ala Met Pro Gly Asn Val 340
CAG Gln 260	GGC CTT Gly Leu 275	GGA CAA AGC Gly Gln Ser	AAG Lys	TAC AAG Tyr Lys 325	ATG (Met 1340
TTC		GGA Gly	CAG Gln	GGC 61y 6	GCG 1
	CCT	TGG Trp 290	GTG Val	cag Gln	TGT (Cys)
Arg	TTG	GCC	GGG Gly 305	Pro (GAA 1 Glu (
·	ហ	10	15	20	

GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG

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Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu 420

1104	1152	1200	1248
CCC	TGT Cys	ACA Thr 400	GGT Gly
GGT	CTG	ACC	TGG
TTG	AGC	CTG	GCC
AGC Ser 365	AAG	CCT (AAA (Lys ?
CAT His	GAG Glu 380	CAC (GGT A
GGT	GAG Glu	CAG (Gln 1 395	GTG C
CCC	CC.A Pro	TGC Cys (
CCG	GAC AAA ASP Lys	CAG C	TGT AGT Cys Ser
TGC Cys 360	gac Asp	CAC His (TGC 1 Cya (
GTC	ATT GCC GAC AAA Ile Ala Asp Lys 375	GAA Glu 1	TGC 1 Cys (
TGT Cys	ATT Ile	ACC Thr 390	CTC 7
CGC	TGC	AGC Ser	CAG (Gln I
TAT Tyr	CAG Gln	GTG Y	CGC (
TCT Ser 355	GCA	CTT (ACC CGC CAG CTC TGC Thr Arg Gln Leu Cys
GGC	GCA Ala 370	CGC (CTA A
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1344	•	1
CA CCA		
CCT CAC	Pro	
GTA CCA TAT	Val Pro	440
TGG GAA AGG	Trp Glu	
ATC TGC CCC GGC	Cys Pro Gly	435

-173-

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1392	1440	1488	1536	1584	1632
GCT CAC CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp 450	GGG CCA CCC AAA CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro 465	CCC CTC GAG GAC ACA GAG GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro 485	GTG AGT GAG GGA TCG GTG CAG CAG AGC CAC CCC ACT ACC ACC ACC ACC ACL Val Ser His Pro Thr Thr Thr Thr 500	TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC TCT CGC CCC TCC CCA CCT Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro 515	ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC CGA AGT GCA GTG Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val 530
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1680	1728	1776	1824	1872	1920
AAC Asn 560	TAC Tyr	TAC	AAA Lys	CGA	CTG Leu 640
TTG	GAT ASP 575	CGC	366)	ABC (GAC (Asp I
CGA Arg	TCG	CAC His 590	CCC GGG AAA Pro Gly Lys	TGC AAC CGA Cys Asn Arg	GTG G Val A
ТСУВ	CCC	CAG Gln	TGC GGC CCC GGG Cym Gly Pro Gly 605	TGT CAC TGC AAC Cys His Cys Asn 620	TGC GTG GAC Cys Val Asp
GAG Glu	GGC	CCG	TGC (Cys)	TGT (Cys 1 620	
GAT Asp 555		CAC	CCC TGC Pro Cys	AAT 7 Asn (CGC 1 Arg 8 635
ACC	GTG Val 570	TCA CAC Ser His	GAG Glu	TCC TAC AAT Ser Tyr Asn	CTC CAC GTG GGT GCA GGG GGC CGC TCG Leu His Val Gly Ala Gly Gly Arg Ser 630
GAG	TGT Cys	CGG Arg 585	GAT GTG AAC GAG TGC GAG GCA GAG Asp Val Asn Glu Cys Glu Ala Glu 595	Ser 7	3GG 0
ACA	CAG	TAC	GAG Glu 600	3GC 3	sca c
GTC	GGA	GGC .	TGC Cys	GGT GGC Gly Gly 615	3GT (
CAG Gln 550	CAT His		Gla	Act o The O	GTG C Val G 630
ACT	ддс G1у 565	AAC Asn	AAC	AAC ACT GGT GGC Asn Thr Gly Gly 615	AC G
CCC	тст	TGC Cys 580	GTG Val	ATG 1	TC C
GCC	ATC Ile	CAC	GAT (Asp 7595	TGT 1	CGC (Arg I
ATC	AAT Asn	TGC Cys	GTT (Val)		TAC C Tyr A
GAG Glu 545	CAG AAT Gln Asn	rcc . Ser (TGT (GGC ATC Gly Ile 610	GGC T Gly. T 625
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9	2016	2064	2112	2160	2208
ile	CTC	GAC Asp	TTC	GCC Ala 720	GGA
Phe Cys Ile 655	CGG Arg	CGC	AGC	CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala 715	CCT GGA Pro Gly 735
Phe	TAC Tyr 670	TGT	GGC G1y	GGC (TCT (Ser E
61y	GGC Gly	GAG Glu 685	CCT Pro	3GG (rgc 1
G1y	CCT	GAC	AAA CCT Lys Pro 700	che eee Gln ely) o.z.
Cys Gly Asp Gly 650		ATC GAC Ile Asp	ABD	AGC Ser (715	ACC CCC TGC Thr Pro Cys
G1y 650	TGC AAC TGC TAT Cys Asn Cys Tyr 665	TGC GAA GAC ATC GAC GAG Cys Glu Asp Ile Asp Glu 680	GAA AAC Glu Asn	CGT	AAC GAA TGC TCC GAA GGT ACC CCC TGC Asn Glu Cys Ser Glu Gly Thr Pro Cys 725
Cys	GGT CAC TAC AAA TGC AAC Gly His Tyr Lys Cys Asn 660	GAA GAC Glu Asp	rgr Cys	TAC	AAC GAA TGC TCC GAA GGT Asn Glu Cys Ser Glu Gly 725
Leu	TGC	TGC Cys 680	GGC AAA TGT Gly Lys Cys 695	GGC .	rcc c
His	лал Гув	ATT Ile	GGC . G1y :	CCT Pro (rgc 1
Pro	TAC	CCC	GAT Asp	CAG (Gln 1710	3AA 1
Lys 645	CAC His	CCG Pro	CCT GAT Pro Asp	TGC Cys C	AAC (Asn (
Cys Ala Lys Pro His	GGT Gly 660		TGC	GCC Ala	
Сув	CCT	TCC CGA Ser Arg 675	ACC	ATC GCC TGC Ile Ala Cys	GAT GTC Asp Val
Asn Glu Cys Ala Lys Pro	TTC	GCC	AGC Ser 690	TGC 1 Cys 1	CGT C
Asn	AAC	AAG Lys	CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys 690	AAG 1 Lys C 705	TGT C Cys A
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Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro 820 825

2256	2304	2352	2400	2448	2496
TGT GCC CAG GGG Cys Ala Gln Gly 750	TGT Cys	GGC Gly	CGG Arg 800	ATC	CCC
CAG Gln	gac Asp	CCA	gat Asp	TGC Cys 815	
GCC Ala 750	GAT Abd	ACA Thr	AGG Arg	GCC	CTC TGT
TGT Cys	GTG Val 765	AAC Asn	Ser		GT C
ACG Thr	GAC Asp	ACG AAC ACA Thr Aen Thr 780	CTG TCA AGG GAT Leu Ser Arg Asp	CCT GCG GCC Pro Ala Ala	GA 1
TGC Cys	CTC AGT IGC ATA GAC GIG GAT Leu Ser Cys Ile Asp Val Asp 760	CAA GAT GGC ATC TGC ACG AAC ACA Gln Asp Gly Ile Cys Thr Asn Thr 775	CAT (His)	TTC (Phe P	TGC ATC AAT ACC AAT GGT TCC TAC AGA TGT
TAC CGT Tyr Arg 745	ТGC Сув	ATC Ile	TAT Tyr 1	GAC 1 ASP 1 810	נכט ז
TAC Tyr 745	CTC AGT IGC Leu Ser Cys 760	GGC	TCC GGC TAT Ser Gly Tyr	GAA TGT GAC Glu Cys Asp 810	GT 1
TCT	CTC Leu 760	gat Asp	Ser (GAA 3 Glu (AT G
GGT	CGC	CAA GAT Gln Asp 775	CTC TCC Leu Ser	GAT ((CC)
CCG GGT Pro Gly	GGA	TGC	TGC (Cys 1 790	ATT C	AT A
	ACC CGC ACA GGA CGC Thr Arg Thr Gly Arg 755	GTG	CAG 3	GAG GAC ATT Glu Asp Ile 805	TC A
AAA CTT Lys Leu 740	CGC	GGG AAA GTG Gly Lys Val	TGT (GAG G Glu A	GC A
GAG Glu	ACC Thr 755	GGG AAA Gly Lys	cag : Gln (TGT C Cys 6	GAC I
TGT	CGA	GCT (Ala (TTC (CGC 1	GGT G
TGG	ATA (GAG C	TCT I Ser P 785	AGC C Ser A	GGG GGT GAC TGC ATC AAT ACC
	rv	10	15	50	

2544				2592				2640			2688				2736				2784	í	:
GAT	Asp			AAC	Asn			ACC	Thr	880	AAG	Lys			TIG	Leu			299	Glv	
ATA	Ile			GAG	Glu			CTC	Leu		AAG	Lys. Lys	895		GIA	Val			GCT		
GAT	Asp			TGC	Сув			ACA	Thr		CAC AAG AAG	His		1	AGC	Ser	910	2	GGA	Leu Gly Ala	•
AAA	Lys Asp	845		ပ္ပင္ပ	Ala			TTC	Phe			His 1		i	SAC 7	Asp S		• .	OTO O	en G	925
AAG	Lys			CCC CAT GCC	His	860		GGT	Gly		ညည	Pro 1			TGT GAC	Cya 1			TCT	Ser I	01
TGC	Сув			C C C	Pro			GAG	Glu	875	CAG CCC CAC	Gln				Phe (TGC 1	Cys S	
CAT CGG TIG GIG GGC GGC AGG AAG TGC AAG AAA GAT	Lys		-	CIG	Leu			GAT	Asp		GAG	Glu	890	Ş	516	Val			TGT 1	Cys	
AGG	Arg			TGC	Сув			TGT	Сув		GTG	Val (ACA GIG	Thr	905	•	. DD:	Сув	
ggG	Gly Gly Arg	840	-	כוני	Leu		•)	GTC	Val Cys Val Cys		GAG GAG GTG GAG	Glu		ç	SAC	Asp 7	Ο,		CAG GAA TGC	lu c	920
gg	$_{\rm Gly}$			<u>က</u>	Gly Leu	855		TGT GTC	CyB		GAG	Glu (E	GAT GAC	Asp 1			AG O	Gln Glu	
GTG	Val		. (3	Pro			GIC	Val	870	TGT	Сув				Phe 1			CAG	Gln (
TTG	Leu Val		į	CAG GAC	Asp			TAT	Tyr		වුවු	Gly	885		ر 1	Asn			ACT (Thr (
9	His Arg		5	3	Gln			TCC	Ser		CAT	His		į	7 7 7 7	Leu Asn	900		FIC 1		
		835	Ç	AG	Ser			ည္သ	Gly		CAG	Gln		, C&E	ואר	Tyr]	•		AAT GTC	Asn Val	915
CTG GGT	G1y		Ş	7	Сув	850		CAG	Gln		GAC	Asp		ניים	ָ כַּ	Сув			ACC 1	Thr 1	0.
CIG	Leu		ţ	5	Glu			CIC	Leu	865	CAG	Gln		טעט		Glu			GCT 7	Ala 1	
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2832	2880	2928	2976	3024	3072
TCA GCC Ser Ala	GGA CAA Gly Gln 960	GAA TGC Glu Cys 975	C TCG	r GGC p Gly	r Aac r Asn
TAC AGC T Tyr Ser S	TCA	GAC ATC GAC GAA TGC Asp Ile Asp Glu Cys 975	TGT GTG AAC Cys Val Asn 990	TAC TAC GAT Tyr Tyr Asp 1005	T GAG TCT p Glu Ser
CCA GTC TAC AGC TCA GCC Pro Val Tyr Ser Ser Ala 940	CTA	CGT GAC A Arg Asp I	GGC AAG TV Gly Lys Cy	TTC	GAG TGC TTG GAT GAG Glu Cys Leu Asp Glu 1020
CCC TGT Pro Cys	ggg aaa gly lyb	GCC CAC Ala His 970	TGC AAG GAG GGC AAG TGT GTG AAC Cyb Lyb Glu Gly Lys Cyb Val Asn 985	CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly 995	GAC GAG TC Asp Glu C)
GAA ATC TAT Glu Ile Tyr 935	CCT	ATT CCT Ile Pro	ATC TGC Z	TGC TAC TGC AAG Cys Tyr Cys Lys 1000	TGC GTG GAC GTG GAC GAG Cys Val Asp Val Asp Glu 1015
TGC GAA Cys Glu	CTG GTG Leu Val	GAA CTA TGC Glu Leu Cys 965	GCA GAG Ala Glu	GAG TGC	TGC GTG (Cys Val)
TGG GGA GAC CAC TGC GAA ATC Trp Gly Asp His Cys Glu Ile 930	r CAC AGC CTG His Ser Leu	TGT Cys	TTT	CCC GGC TAC Pro Gly Tyr 995	GAG
TGG GGA Trp Gly 930	GAA TTT Glu Phe 945	CAA CAT Gln His	ATA TTG Ile Leu	CAG CCC Gln Pro	AAC CTG CTG Asn Leu Leu 1010
	Ŋ	10	15	20	. ,

3120	3168	3216	3264	3312	3360
TGC AGG AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC CyB Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys 1025	ACT CCG CCG GCA GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG ATC CCG Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro 1045	GAG AGA TGG AGG ACC CAG AGA GAC GTG AAG TGT GCT GGG GCC AGC Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser 1060	GAG GAG AGG ACG GCA TGT GTA TGG GGC CCC TGG GCG GGA CCT GCC CTC Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu 1075	ACT TTT GAT GAC TGC TGC CGC CAG CCG CGG CTG GGT ACC CAG TGC Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys 1090	AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC CCG ACT TCA CAG Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln 1105
	ហ	10	15	7	

3408	3456	3504	3552	3600	3648
AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG GGG AAG Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys 1125 1135	TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg 1140	TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu 1155	TGT CCT GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp 1170 1175	ATT GAT GAG TGC CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser 1185	GAG CGG TGC GTG AAC ACC AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala 1205
	Ŋ	10	15	20	

-181-

3696	3744
GGC TTC ACG CGC AGC CGC CCT CAC GGG CCT GCG TGC CTC AGC GCC GCC Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala 1220	GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA GTG ATC GAT CAT CGA GGG Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly 1235

(2) INFORMATION FOR SEQ ID NO:3:

TAT TTT CAC Tyr Phe His

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1251 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu		Gln	Pro	Cya	Thr 80	Pro	Pro	31,
Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Leu Ala Leu	15	Ala	Ala	Ser	Ser	Pro Leu Pro 95	Сув	Gln Val Pro Ala Ala Gly Thr Gly
Leu		G1y 30	Phe	Asp	His	Pro	Leu 110	Gly
Leu		Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala 20	Gln Arg Phe Lys Val Val Phe 40	Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg 55	Gly	Сув	Asn Gln Cys Leu 110	Ala (
Leu		Gly	Val	Сув 60	Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly 65 75	Val	Gln	Ala i
Leu		Pro	Lув	Gln	Glu 75		Asn	Pro ;
Ala	10	Arg	Phe	Gly	G1γ	V al 90	Arg	Val
Leu		Gly 25	Arg	Глув	Ile	Phe Arg Val Val 90	Ser Arg 105	gln .
Leu		Val	Gln 40	Leu	Leu	Phe	Ser	Сув
G1y		$\mathfrak{G}1_{\mathbf{y}}$	Gly Ala Gly Arg Trp Ala 35	Сув 55	Thr	Ala	Суз	Phe Cys
Leu		Arg	Trp	Thr	Met 70	Ser	Gln	Arg
Ala	S	$_{ m G1\gamma}$	Arg	Arg	Asn	Leu Thr Gly Ser 85	Gly	Gly Arg
Ala		G1y 20	Gly	Lys	Ser	Thr	Gly 100	Thr
Gln		Pro	Ala 35	Сув	Gly	Leu	Asn	Phe
Met Arg		Leu Gly		Ile Cys 50	Gln	Thr	Met Asn Gly Gly Gln 100	Asp
Met.	-	Leu	Ala	Val	Gln 65	Asp	Сув	Pro
		ហ		10	51		20	

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Ser	Ser 160	Pro	$_{ m G1y}$	Asn	Ile	Pro 240	$Gl_{\mathcal{Y}}$
Met	Ala	Gly 175		Val	Arg	Leu	Leu (
Ala	Val	Pro Gly 175	Pro Leu 190	Val	His	Leu 1	Pro Leu
Arg	Ser	Pro	Val	Pro .	Val	His 1	
Asp 140	Glu	Asp	Leu Val	Pro	Gln Val 220	Gln)	Gln Lys
Pro	Gly 155	Ile Ala 170	Phe	Pro	Val	Ser 235	
Ser Gly Pro Gly Trp Pro Asp Arg 135	Pro Glu Gly Glu 155		Ala		Ser	Ser	Pro Thr 250
Gly		Val	Ala 185	Gln	Ala		or.
Pro	Ala	Glu	His	Val 200	Glu	Pro	Arg 1
Gly 135	Pro Leu Ala 150	Val	Gln	Glu Val Gln Ala 200	Pro Pro Glu Ala 215	Glu Gly Pro Ala 230	Pro Arg Pro
Ser	Pro 150	Ala	Ala		Pro	Glu (His 1
Ser	Pro	Tyr 165	Pro	Ser Ala		Ala	Pro His 245
Gly Thr Gly Ser 130	Pro Leu	Ile	Pro 180		His His	Pro Asn Ala	Pro
Thr	Pro	Ala	Gly	Gln 195	Val	Pro	Lys
	Thr Gly 145	His	Gly Glu Gly Pro Pro Ala 180	Gly Gln Ile 195	Arg 210		Pro Lys
Ala	Thr 145	Lys	Gly	Pro	Val	Glu Gly 225	His
	rv		10	r.		20	

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Pro	Thr	Thr	Сув 320	Asn	Asn	Pro	Cya
Asn	Gly	Tyr	Asp	11e 335	Leu Asn Asn 350	Pro Gly His Ser Leu Gly 365	Ser Leu
Ser 270	11e	Gln	Ala	Asp	Leu 350	Leu	Ser
Gly	Ser 285	Gln Leu 300	Gly Ala	Gln Asp	Сув	Ser 365	Lув
Суз	G1y	Gln 300	sly Glu Val 315	CyB	Gly Asp	His	Pro Glu Glu Lys
Pro	CyB	Pro	Glu 315	His	Gly	Gly	Glu
Gln	Сув	Сув	Sly	Thr 330	His	Pro	Pro
Lys 265	Авр	Lys	Arg	Ser	Сув 345	Pro	Lys
Pro	Gln Glu Asp 280	His	Val			Сув 360	Ile Ala Asp Lys
Leu	Gln	Сув 295	Pro	Leu Asn	Asn Val		Ala
Thr	Lys	Lув	Val 310	Arg	Pro Gly	Cys Val	Ile
Gln Asp 260	Leu Thr	Ser	Pro	Lys 325	Pro	Arg	
Gln 260	Leu	Gln	Ьув	Tyr	Met 340	TYT	Ala Ala Gln Cys
Phe	G1y 275	Gly	Gln Lys	Gly Tyr	Ala	Ser Tyr 355	Ala
Сув	Pro	Trp 290		Gln	Сув	Gly	Ala
Arg	Leu	Ala	Gly val 305	Pro	Glu	Pro	ren
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Thr 400	Gly	Glu	Asp	Asp	Pro 480	Pro	Thr
Thr	Trp 415	Ьув	Pro Asp	Pro	Ala	Pro 1	
Pro Leu	Ala	Phe 430	Pro	Ala	Arg	Asp	Pro Thr Thr Thr 510
Pro	б1у ьув	Ala	Leu 445	Pro	Ser Arg	Met	Thr
нів	Gly	Ala	нів	Leu 460	Pro	Thr	Pro '
Gln 395	Ser Val 410	Thr	Pro	Pro	Ser 475	Val	His
Сув	Ser 410	Asp Gly 425	Tyr		Glu	Glu Arg Gly Val	Ser
Gln	Cys	Asp 425	Cys Pro Gly Trp Glu Arg Val Pro 435	Leu Leu	Pro	Arg	Gln Gln Ser 505
Glu His	Суз Сув	Pro Ala	Val 440	Arg	Leu	Glu	Gln (
G1u			Arg	Pro Gly Gly Lys Arg 455	Pro Gln Gln Leu 470	Glu	Val
Thr 390	Gln Leu 405	Сув	Glu	Gly	Gln 470	G1 u	Ser
Ser		Gln Arg 420	Trp	Gly	Pro	Thr 485	Arg
Leu Val	Arg	Gln 420	Gly	Pro	Lys	Авр	Glu Arg 500
Leu	Thr	Сув	Pro 435	нів	Pro	Glu Asp	Glu
Phe Arg 385	Leu	Arg	Сув	H18 450	Pro	Leu	Ser
Phe 385	Arg	Ala	11e	Ala	Gly 465	Pro	Val
	Ŋ		10	15		70	·

Pro	Val	Asn 560	Tyr	Tyr	ьув	Arg	Leu 640
Pro	Ala	Leu	Asp '	Arg '	Gly]	Asn Arg	Asp Leu 640
Ser	Ser	Arg	Ser	His 590	Pro Gly	Сув	Val
Pro 525	Ser Arg 540	Сув	Pro	Pro Gln His 590	G1y 605	His	Cys Val
Arg	Ser 540	Glu	Cys Val Pro Gly 570	Pro	Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly 595 600	Tyr Asn Cys His 620	
Ser	Pro Pro	Asp 555	Pro	Cys Asn Ala Gly Tyr Arg Ser His 580	Pro	Asn	Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser 625 635
Pro Glu Leu Ile 520	Pro	Thr	Val 570	Ser	Glu	Tyr	Gly
Leu	Pro Asp Leu 535	Glu	Сув	Arg 585	Ala	Gly Ile Cys Met Asn Thr Gly Gly Ser 610	Gly
Glu 520	Asp	Thr	Gln	Tyr	Glu 600	σ1у	Ala
Pro	Pro 535	Gln Val 550	Gly His Gly Gln 565	Gly	Сув	G1y 615	Gly
Tyr	Геи	Gln 550	His	Ala	Glu	Thr	val 630
Pro	Phe	Thr	G1y 565	Asn	Asn	Asn	His
Pro Arg 515	Arg	Pro	Cya	Сув 580	Val	Met	Leu
Pro 515	His	Ala	Ile	Cys His	Авр 595	Сув	Arg
Pro	Phe 530	Ile	Gln Asn	Сув	Val	Ile 610	Tyr
Ser	Thr	Glu 545	Gln	Ser	Сув	Gly	G1y 625
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Ile	Leu	Asp	Phe	Ala 720	Gly .	Gly	Cys
Су <i>в</i> 655	Arg	Cys Arg	Ser	Ser Gln Gly Gly Gly Ala 715	Pro Gly 735	Tyr Arg Cys Thr Cys Ala Gln Gly 745	
Phe	Tyr 670	Суз	Pro Gly	Gly	Ser	Ala 750	Ile Asp Val Asp Asp 765
Gly	Pro Gly	Glu 685	Pro	Gly		Сув	Val 765
Gly		Asp	Lys 700	Gln	Pro Cys	Thr	Asp
Gly Asp Gly Gly 650	T _Y T	Ile	Asn	Ser 715	Thr	Сув	Ile
G1y 650	Ç	Glu Asp	Glu Asn Lys 700	Arg	G1y 730	Arg	Cys
Cya	Asn 665	Glu	Сув	Tyr Arg	Aen Glu Cys Ser Glu Gly Thr 725	Tyr 745	Ser
Leu	Суз	Сув 680	Lyв	Gly	Ser		Leu 1
Нів	Lyв	Ile	Gly Lys 695	Pro	Сув	Gly .	Arg)
Pro	Tyr	Pro Pro	Asp	Gln Pro Gly 710	Glu	Pro Gly Ser	Gly Arg Leu Ser 760
Lys 645	Gly His 660	Pro	Pro	Сув	Aen 725	Leu	
Ala	G1y 660	Arg	Сув			Lys 740	Arg Thr
Сув	Pro	Ser 675	Thr .Cys	Ile Ala	Asp val	Glu Lys Leu 740	Thr 755
Glu	Phe	Ala	Ser 690	Cy8	Arg	රු	Arg
Asn	Asn	Lув	Pro	Lys 705	Cys	Tr	Ile 1
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Gly	Arg 800	Ile	Pro	Asp	Asn ·	Thr 880	ьув
Pro	Авр	Cys 815	Cyв	Ile	Cys Glu Asn	Leu	Lув 895
Thr	Arg	Ala	Leu 830	Авр	Cys	Thr	His
Asn	Ser	Ala	Сув	Lys Asp 845	Ala	Phe	His
Thr 780	Leu	Pro	Arg	Lya	Pro His Ala 860	Gly	Pro His
Сув	H18 795	Phe	Tyr	Сув	Pro	Glu 875	Gln
Ile	Tyr	Asp 810	Ser	Ьγв	Leu	Cys Asp Glu 875	Glu 890
Gly	Gly	Сув	Gly 825	Arg	Cys	Cys	Val
Gln Asp 775	Ser	Glu	Asn	G1y 840		Val	Glu
	Cys Leu 790	Asp	Thr	Gly	G1y 855	Сув	Glu Glu Val
Сув	Сув 790	Glu Asp Ile Asp Glu 805	Asn Thr	His Arg Leu Val Gly Gly Arg 835	Pro Gly Leu 855	Val 870	Сув
Val	Gln	ASP 805	Ile	Leu	Asp	Tyr	Gly 885
Gly Lys Val	Сув	Glu	Сув 820	Arg	Gln Asp	Ser	
Gly	Gln	Сув	Авр	His 835	Ser	Gly	Gln His
Ala 770	Phe	Arg	біу біу Авр	Gly	Сув 850	Gln	Asp
Glu	Ser 785	Ser	G1y	ren	Glu	Leu 865	Gln Asp
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Leu	$_{ m G1y}$	Ala	Gln 960	Сув	Ser	Gly	Asn
Phe Asp Asp Thr Val Phe Cys Asp Ser Val 905	Ser Leu Gly Ala Gly 925	Ser Ala	Gly Gln 960	Asp Glu Cys 975	Asn Ser	Asp	Ser
Ser 910	G1y	Ser	Ser	Asp	Val 990	Tyr	Glu
Asp	Leu 925	Tyr		Ile	ر ک ھ .	Tyr Tyr 1005	Asp (
Сув	Ser	Pro Val Tyr 940	Arg Leu His 955	His Arg Asp 970		Phe	Cys Leu Asp Glu
Phe	Cys		Arg 955	Arg	С1у Був	Gly	Сув
Val	Сув	Pro Cys	Pro Asp Gly Lys	Нів 970	Glu	Gln	glu (
Thr 905	Glu Cys 920	Pro	Gly	Pro Ala	Lys 985	Cys Lys Gln 1000	Asp Val Asp Glu
Авр	Glu 920	Tyr	Asp	Pro	Сув Lys 985	Сув 1000	Val
Asp	Gln	Ile 935	Pro	Ile	Ile	17.	Asp .
	Gln	glu	Val 950	Сув	Glu Ile	ζ γ в	Val
Leu Asn 900	Thr	Cys	Leu	Leu Cys 965	Ala	Glu	CyB
	Asn Val 915	His	Ser	Glu	Gly Ala 980	Tyr	glu
Tyr		Asp 1	His	Сув	Phe	Gly 995	Leu
Сув	Thr	Gly 930	Phe	нів	Leu	Pro	Leu Leu Glu 1010
Glu	Ala	Trp	Glu 945	Gln	Ile	G1n	Asn
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Cy 100 100 Glu 1100 1100 1100 1100 1100 Ser Ser Ser	Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys 1025 1035	r Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro 1045 1050	Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser 1060	Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu 1075 1080	Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys 1090 1095	Arg Pro Ja Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln 1105 1110	Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys 1125 1135	Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg 1140 1145
	Cys Arg 1025	Thr	Glu Arg	Glu	Thr	Arg Pro 1105	Ser	Ser Pro

Glu	
Сув	
Val	
Ala	776
Gly	
Gly	
Pro	
Arg	
Pro	1150
Val	
Сув	
Pro	
Gly	
Ser	1155
Val	
Сув	

Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp

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Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser 1170

1195 1190 1185

Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala 1210 1205 10

Gly Phe Thr Arg Ser Arg Pro. His Gly Pro Ala Cys Leu Ser Ala Ala 1220

15

Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly 1235

Tyr Phe His

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(2) INFORMATION FOR SEQ ID NO:4:

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. 5	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
	(ii) MOLECULE TYPE: DNA (genomic)	
10	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
	AACATGACGC TCATCGGAGA GAAC	24
	(2) INFORMATION FOR SEQ ID NO:5:	
15	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
20	(5) Iolobodi. Illiear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:	
25	AGGTGATCGC AGATCCTC	18
	(2) INFORMATION FOR SEQ ID NO:6:	
30	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
35	(ii) MOLECULE TYPE: DNA (genomic)	
	(Jenomic)	

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	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
	TACCGATGCT ACCGCAGCAA TCTT	24
5		
	(2) INFORMATION FOR SEQ ID NO:7:	
	(i) SEQUENCE CHARACTERISTICS:	
10	(A) LENGTH: 22 base pairs	•
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
15	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:	
20	ATGCCTAAAC TCTACCAGCA CG	22
20	ATGCCTAAAC TCTACCAGCA CG	22
20	ATGCCTAAAC TCTACCAGCA CG (2) INFORMATION FOR SEQ ID NO:8:	22
20		22
20	(2) INFORMATION FOR SEQ ID NO:8:	22
-	(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS:	22
-	(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs	22
-	(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE: nucleic acid	22
-	(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	22
25	(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	22

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	(2)	INFORMATION	FOR	SEQ	ID	NO:9:
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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
- 5 (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGTCCAAGTT GTGTCTTAGC AG

22

- (2) INFORMATION FOR SEQ ID NO:10:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 53 amino acids
- 20
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly 10

Gln Thr gly Glu Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro 30

25

Lys Gly Glu Thr Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala

10

45

Gly Glu Glu Gly Lys

(2) INFORMATION FOR SEQ ID NO:11: 15

SEQUENCE CHARACTERISTICS: (I)

(A) LENGTH: 159 base pairs

TYPE: nucleic acid (B)

STRANDEDNESS: single ij

20

TOPOLOGY: linear <u>(a)</u>

(11) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

09	120	159
GACGGGTGAG	Accrecrese	
GGCCCTCCCG GTCCTCAAGG TGCAACTGGT CCTCTGGGCC CCAAAGGTCA GACGGGTGAG	CCCGGCATCG CTGGCTTCAA AGGTGAACAA GGCCCCAAGG GAGAGACTGG ACCTGCTGGG	
ccrcreeecc	GGCCCCAAGG	GAAGGAAAA
TGCAACTGGT	AGGTGAACAA	TGCTGGTGAA
GTCCTCAAGG	CTGGCTTCAA	CCCCTGGCCC
GGCCCTCCCG	CCCGGCATCG	CCCCAGGGAG CCCCTGGCCC TGCTGGTGAA GAAGGAAAA
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10 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:(A) LENGTH: 1442 amino acids(B) TYPE: amino acid(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(D) TOPOLOGY: linear

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

							:
Leu	Gly	Lys	Сув	Pro 80	Ser	Ile	Asp
Leu 15	Ala	Trp	Leu	Ile	Ala 95	Asp	31y 1
Leu Val Leu Leu Thr Leu Leu 10	Gln Glu 30	Val	Val		Cys Pro Ala Asp Leu Ala Thr Ala 90	Leu Gly Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile 100	Asp Gly Pro Ala Gly Glu Gln Gly Pro Arg Gly Asp Arg Gly Asp
Leu	Gln	Asp 45	Asn	Pro	Ala	Pro (Asp 7
Leu	Cys Gln Gly Gln Asp Ala 25	Lys Asp Lys Asp Val 45	Thr Gly Asn Val 60	Leu Asn Pro Glu 75	Leu	G1u	317 7
Val	Asp	Asp	Thr	Leu 75	Asp	Gly (Arg (
Leu 10	Gln	LyB	Сув Авр	Glu Asp Pro Asp Cys 70	Ala 90	LyB	Pro
Ser	G1y 25	Gln Asn Gly Gln Arg Tyr 40	Cya	Asp	Pro	Gln 105	Gly
Gln	Gln	Arg 40	Сув Val 55	Pro	Сув	Gly	Gln
Pro	CyB	Gln	Сув 55	Asp		Г уя	Glu
Leu Gly Ala 5	Val Leu Arg 20	Gly	Ile	Glu 70	Pro Ile	Pro	Gly
G1y 5	Leu	Asn	Cys Arg	Ile Cys	Сув Сув 85	Gly	Ala
Leu	Val 20	Gln	Сув		Сув	Leu 100	Pro
Arg	Ala	Leu 35	Ser	11e	Gly Glu	Lys	Gly 115
Ile	Ala	C. YB	Ser 50	Asp	Gly	Gly Arg	Asp
Met 1	Ile	Ser	Pro	Asp 65	Phe	Gly	Arg

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Phe Ala Ala Gln Met Ala Gly Gly Tyr Asp Glu	Gln Met Gly Val Met Gln Gly Pro Met Gly Pro	Pro Arg Gly Pro Pro Gly Pro Ala Gly Ala Pro Gly Pro Gln	Gly Phe Gln Gly Asn Pro Gly Glu Pro Gly Glu Pro Gly Val Ser Gly	Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Lys Pro Gly Asp	Gly Lys Ser Gly Glu Arg Gly Leu Pro	Phe Pro Gly Thr Pro Gly Leu Pro Gly	Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu
135	150	165 175	180	195	220	235	245
Lys Gly Glu Lys Asn Phe 1	Lys Ala Gly Gly Ala Gln M	Met Gly Pro Arg Gly Pro P	Gly Phe Gln Gly Asn Pro G	Pro Met Gly Pro Arg Gly Pr	Asp Gly Glu Ala Gly Lys Pro Gly Lys	Gly Pro Met Gly Ala Arg Gly	Val Lys Gly His Arg Gly Tyn
130	145	165		195	210	225	245

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Asn	Gly	Gln	Pro 320	Gly	Asn	Asp	dly
Glu		Gly	Gly	Thr 335	Gly	Thr	Ala
G1y 270	Glu	Asp	Gly	Pro	Pro 350	31y	11e
Pro	G1y 285	Asn	Ala	Gly	Glu	Pro (31y :
Ala Gly Ala Pro Gly Val Lys Gly Glu Ser Gly Ser Pro Gly Glu Asn 260 270	Pro Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg 275	Thr Gly Pro Ala Gly Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln 290	Pro Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro 310	Pro Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly 325 335	Pro Glu Gly Ala Gln Gly Ser Arg Gly Glu Pro Gly 340	Ser Gly Asn Pro Gly Thr Asp 365	Pro Gly Ile Ala Gly 380
G1y .	ren	Arg	Gly 315	Glu	Arg	Gly	Ala
Ser	вιу	Ala	Val	G1y 330	Ser	Ser	Gly Ile Pro Gly Ala LyB Gly Ser Ala Gly Ala 370
G1u 265	Arg	Gly	Pro	ьув	G1y 345	Ala	Ala
Gly	Pro 280	Ala	Gly	Ala	Gln	Pro Gly Pro Ala Gly Ala 360	Ser
Lув	Gly	Ala 295	Pro	Gly	Ala	Ala	G1y 375
Val	Met	61y	Pro 310	Pro	Gly	Pro	Lys
Gly.	Pro	Ala	Gly	Ala 325	Glu	Gly	Ala
Pro 260	Gly	Pro	Ala	Gly	Pro 340	Pro	б1у
Ala	Pro 275	Gly	Pro	Pro	Gly	Ser 355	Pro
GIY	Ser	Thr 290	Pro Gly 305	Phe	Arg	Gly	11e 370
Ala	Gly	Arg	Pro 305	Gly	Ala	Pro	Gly
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Ala 400	Ala	Gly	Ala	Arg	Gly 480	Pro	Pro
Ala Pro Gly Phe Pro Gly Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala 385 395 400	Ile 415	Pro Ala 430	Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala 440	Ile Gly Pro Pro Gly Glu Arg 460	Pro Gly Gln Asp Gly Leu Ala 475	Ser Gly Leu Ala Gly Pro	Pro Gly Leu Pro . 510
Gln	Gly	Pro 430	Arg	Gly	Leu	Ala	G1y 510
Pro	Pro	Gly	Lys 445	Pro	Gly	Leu	Pro
Gly	Gly Gln Ala Gly Glu Pro Gly 410	Gly Glu Thr	Gly	Pro 460	Asp	Gly	Glu
Pro 395	Gly	Glu	Glu	Gly	Gln 475	Ser	Gly Glu
Pro	Ala 410	$_{\rm G1y}$	Glu	Ile	σ1у	Pro 490	Pro
$_{ m G1y}$	Gln	Pro Lys 425	Gly	Pro		Gly	Arg 505
Arg	Gly		Ala 440	Gly	Phe	Arg	Gly
Pro	Lув	б1у	Pro	Ala 455	Gly Phe	Pro Gly Glu Arg 485	Pro
Gly 390	Pro	Gln	Gly	Gly	Arg 470	Gly	Авр
Pro	G1y 405	Asp	Pro	Gly	Asn	Pro 485	Gly
Phe	Gly Pro Leu Gly Pro Lys	Lys Gly Asp Gln Gly 420	Gly Ala 435	Gly Glu Pro Gly Gly Ala Gly Pro 450	Pro Gly	Gly Ala	Ala Asn Gly Asp Pro Gly Arg 500
Gly	Pro	ьув	G1y 435	glu	Pro	Gly	Ala
Pro	ату	Phe	Pro Gln	Gly 450	Ala	Lya	Gly
Ala 385	Thr	Gly	Pro	Arg	Gly Ala 465	Pro	Ьув

Gly	Pro	Pro 560	Gly	Glu	Arg	61 y	Ile 640
Gln Gly	Gly	Pro Gly Pro Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe 545 555	Pro Lys Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly 565 . 570	Gly Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu 580 590	Gly Pro Ala Gly Glu Arg 605	Gly Phe Gln Gly Leu Pro Gly 620	Pro Pro Gly Pro Pro Gly Glu Gly Gly Lys Gln Gly Asp Gln Gly Ile 625 635 640
Pro	Pro	Gly	Glu	ABP 590	Gly	Leu	3ln (
Arg Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly 515 525	Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg 530 530	Met	агу	Lyв	Ala 605	Gly	Авр (
Ala	Gly 540	Val	Ala	Gly	Pro	Gln 620	Gly
Asp	Asp	G1y 555	Lys	Pro	Gly	Phe	Gln 635
Gly	Glu	Pro	Gly 570	Leu	Ser	Gly	Lув
Pro	Gly	Gln	Pro	G1y 585	Pro	Ser	Gly
Arg 520	Pro	Gly	Glu	Arg	Pro Gly 600	Pro Gly Pro Ser 615	Gly
Gly	Ala 535	Arg	Gly	Leu	Pro	Gly 615	Glu
Thr	Gly	A1a 550	Aen	Gly	Pro	Pro	G1y 630
Leu	Ser	Gly	Ala 565	Pro	Gly Ala Ala Gly 595		Pro
Gly	Pro	Gin	Gly	Ala 580	Ala	Gly	Pro
Arg 515	Gly	Pro	Lys	Gly	Ala 595	Gln	Gly
Gly Ala	Val 530	Gly		Leu Ala		Gly Glu Gln Gly Ala 610	Pro
GLy	Lya	Pro 545	Gly	Leu	Thr	Gly	Pro 625
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Arg	Gly	Ala	Gln	G1y 720	Lув	Ala	Gly
Glu 655	Gln	Gly Ala	Leu	ьув	G1y 735	Pro	Ser
в1у	Leu 670		Gly	Pro	Pro	Gly Pro Ala 750	Pro.
Arg	Gly	Pro Lys 685	Pro	31y		Pro (Gly 1 765
Pro	Gln Gly Leu Gln Gly 670	317	Pro 700	Ile Ala Gly Pro 715	31y 1	Pro 1	or C
31y	Ala (Авр Сіу	l YIE	11e / 715	31n (lly I	i Oi
Val (31y 1		lln (Gly 1	Pro G 730	le G	1y P
Pro Gly Glu Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg 655	Pro Gly Ala 665	Pro Gly Thr 680.	Pro Asp Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln 695	la G	Gly Pro Glu Gly Ala 730	Gly Pro Ile Gly 745	Gly Ala Asn Gly Glu Lys Gly Glu Ala Gly Pro Fro Gly Pro Ser Gly 755
ıly ı	er 6	Pro G 680.	ly A	Arg Gly Ala Ala 710	ув С	1y P 7	Glu A
or o	Gly Glu Arg Gly Ser 660	hr F	Pro G 695	1y A	Arg Gly Asp Val Gly Glu Lys 725		1y G
la P	rg G	ly T	70 P	Arg G 710	ly G	T ne	B G
Gly A 645	lu A	6 6	<u>A</u>	u A	લ છ	<u>ጃ</u>	্ন
	<u>5</u>	7 7	ច	9	Val 725	6	GJ
A)	G1y 660	Let	Ast	G1 ,	Asp	Arg 740	Gly
Glu	Pro	Gly Leu Pro Gly Thr 675	Pro	Pro Gly Glu	Gly	dly	Asn 755
Gly	Phe	Arg	G1y 690	Met	Arg	Gly	Ala
Pro	Gly	Pro Arg	Ala Gly 690	Gly 705	Авр	Asp Gly Gly Arg Gly Leu Thr 740	11y 1
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Pro	Pro 800	Gly	Pro	Pro	Gly	Lys 880	Ala
Gly	Gln	Ala 815	Gly	Pro	Pro	$_{ m Gly}$	Arg
Gly Glu Thr Gly 780	Gly	Asp	Gln Gly 830		Pro		Gly Arg Ala
Glu	Asp Gly	Gly Asp	Pro	Gln Gly 845	Gly	Pro Ala	Pro (
G1y 780	Gly Ala 795	Lув	Gly	Ala	Val 860		Pro Pro
Pro	Gly 795	Gly Gln 810	Pro	Gly	Arg	Pro Gly 875	Gly
Glu	Pro	Gly 810	Ala	Arg Gly	Gly	Pro	Ser
Ala Pro Gly 775	Pro	Ala	Gly Ala 825		Ala	Gly	Asp
Pro	Gly	Glu Ala	Ser	Gly Ala 840	Ala	Ala	сіу Авр
Ala 775	Phe Ala 790	азу	Pro	Ьув	Gly 855	Pro Ala	Arg
Gly	Phe 790	Asp Gln Gly 805	бlу	Pro	Pro	G1y 870	Gly Val Arg
Arg	Gly		Pro Gln 820	Gly	Phe	Pro	Gly
Thr Gly Ala 770	Ala	Gly		Thr	Gly	Asn	Ьув
Gly	Pro	ьув	Gly	Gly Val 835	Thr	Gly	Pro
Thr 770	Gly	Ala	Pro	Gly	Ala 850	Asn	Gly
Ser	Pro 785	Gly	Ala	Thr	Gly	Ala 865	Asp
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Val Lys Gly Asp Arg Gly Glu Thr Gly Ala Leu Gly Ala Pro Gly Ala

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Gly	Pro	Arg	G1y 960	Pro	Glu	Gly	
Lyв	σιу	Gln	Pro Gly 960	Gly 975			
Glu 910	Pro	Gly	Glu	Pro	Gly 990	Ma	
Pro Gly Glu Lys 910	Pro 925	Pro	Gly	Pro	Pro	Gly 2 1005	
Pro	Gly Asp Asp Gly Pro Ser Gly Leu Asp Gly Pro Pro Gly Pro 915 925	Gln Gly Leu Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg 930	Pro Ser Gly Glu 955	Gln Gly Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro 975	Gly Pro Ala Gly Glu Pro Gly Arg 985	Pro Gly Ala Asp Gly Pro Pro Gly Arg Asp Gly Ala Ala 995 1006	
Gly Asp Pro Gly Leu Glu Gly Pro Ala Gly Ala 900	Авр	Gly	Pro 955	Arg	Gly	Arg	
$_{ m G1y}$	Leu	Val	Pro Gly Leu Pro Gly 950	Asp 970	Ala	Gly	
Ala 905	Gly	Ile	Pro	Gly	Pro 985	Pro	
Pro	Ser 920	Gly	Leu	Ser	Gly	Pro 1	
Gly	Pro	Arg 935	Gly	Ala		Gly	
Glu	Gly	Gln	Pro 950	Gly	Pro Gly Leu Thr 980	Авр	
Leu	Авр	дју	Phe	Pro 965	Gly	Ala	
G1y 900	Авр	Ala	Gly	Ala	Pro 980	Gly	
Pro	Gly 915	Leu	Glu Arg Gly Phe	Gly	Pro	Pro 995	
Авр	Pro	G1y 930	Glu	Gln	Gly Pro	Ser	
Gly	Glu	Glu	Gly 945	Ьув	Val	Gly	
	ហ		10	r.	1	20	

Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly 1140 1140

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Gln 1040	G1)5	Ast	Arg	$_{ m G1y}$	Pro 1120	Pro
Ьув	Ser Gly 1055	Gly	His	Ser	Gly	Ile 1135
Pro Gly Pro Pro Gly Ser Pro Gly Pro Ala Gly Pro Thr Gly Lys Gln 1025 1040	Gly Asp Arg Gly Glu Ala Gly Ala Gln Gly Pro Met Gly Pro 1045	Pro Ala Gly Ala Arg Gly Ile Ala Gly Pro Gln Gly Pro Arg Gly Asp 1060	Gly Glu Ser Gly Glu Gln Gly Glu Arg Gly Leu Lys Gly His Arg 1075 1080	Gly Phe Thr Gly Leu Gln Gly Leu Pro Gly Pro Gly Pro Ser 1090	Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro 1110	31.y :
Thr	Gly	Pro	Lys (1085	Gly	Pro 1	\sn (
Pro	Met	Gly	Leu	Pro (Gly 1	Ser 1
Gly 1	Pro	Gln	б1у	Pro	Ser (1115	Gly a
Ala	Gly 1 1050	Pro	Arg	Gly	Pro	Asp (
Pro	Gln	Gly 1065	G1 u	Pro	Gly	LyB
Gly	Ala	Ala	Gly (1080	Leu	Ma	Gly
Pro 0	G1y	IJe	Gln	Gly . 1095	Pro	Ser
Ser 1	Ala 5	Gly	gJu	Gln	Gly 1110	Pro
Gly	Glu /	Arg	Gly	Leu	Ser	Gly 1125
Pro	GLy	Ala 1060	Ser	Gly	Ala	Val
Pro	Arg	Gly	Glu : 1075	Thr	$_{ m G1y}$	Pro
61y 5	Авр	Ma	Gly	Phe 7	Gln	σ1у
Pro (Gly	Pro	LyB	Gly	Asp Gln Gly Ala 1105	Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ser Asn Gly Ile Pro 1135
	ın		10	r		70

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg 1395 1400

Lys Ser 1295	y Phe	n Val	n Ile	a Gly 1360	Glu Met 1375	р біу
Ser Ly 12	Gly Gl 1310	ala As	iln As	la Al		Lys As _] 1390
Ser	Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Met Asn Gly Gly Phe 1300 1305	Ser Tyr Gly Asp Gly Asn Leu Ala Pro Asn Thr Ala Asn Val 1315	Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn 1330	Tyr Leu Asp Glu Ala Ala Gly 1355	Gln Gly Ser Asn Asp Val 1370	Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly 1380
îrp Trp	ľhr Met	Pro Asi	3lu Gly 3 1340	Leu Asr 1355	ler Agr	hr Ala
3 Asn ? 1290	, Glu 1 15	ı Ala ı	Thr (Gly s	Tyr 1 5
Pro Arg Lys Asn Trp 1290	the Gly (Asn Let 1320	en Ser	Сув Lyв Asn Ser Ile Ala 1350	le Gln	he Thr 1385
Pro 1	Trp 1	0 Gly 7	1335 1335	Ser I	L Leu I	Arg P
Thr Val 1285	tis Ile	31y Asg	seu Arg	ıye Asn (Asn Leu Lys Lys Ala Leu Leu Ile 1365	ısn Ser
Ala :	3 Lys]	: Tyr (r Phe 1		Lys A	Gly A
Pro Asn Pro Ala	3lu Lyi	Phe Ser 7	Met Th 1330	Thr Tyr His 1345	æu Lys	da Glu
Pro 1	Тув (His I	Gln B	Thr 1	Asn I	Arg A
	v		10		5	20

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1440 Ile Gly Gly Ala Glu Glu Phe Gly Val Asp Ile Gly Pro Val Cys Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp 1420 1435 1415 1430 1410 1425

Phe Leu

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(2) INFORMATION FOR SEQ ID NO:13: 10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGIH: 267 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15

TOPOLOGY: linear <u>a</u>

(ii) MOLECULE TYPE: DNA (genomic)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:13;

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

	AIAGGCCCIT IGGAGACGGC IGITTICCAG ACTCCAAACT AICGIGICAC ACGIGIGGGA	09	
	AATGAAGTGT CTTTCAATTG TGAGCAAACC CTGGACCACA ATACTATGTA CTGGTACAAG	. 021	
'n	CAAGACTCTA AGAAATTGCT GAAGATTATG TTTAGCTACA ATAATAAGCA ACTCATTGTA	180	
	AACGAAACAG TICCAAGGCG CIICICACCI CAGICIICAG AIAAAGCICA IIIGAAICII	240	
ç	CGAATCAAGT CTGTAGAGCT GGAGGAC	267	
3	(2) INFORMATION FOR SEQ ID NO:14:		
	(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 54 amino acida		
15	(B) TYPE: amino acid		
	(C) STRANDEDNESS: single		
	(D) TOPOLOGY: linear		
	(ii) MOLECULE TYPE: peptide	·	
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		:	

Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly
1 5 10 15

Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ala

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Asn Gly 11e Pro Gly Pro 11e Gly Pro Pro Gly Pro Arg Gly Arg Ser

Gly Glu Thr Gly Pro Ala

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(2) INFORMATION FOR SEQ ID NO:15:

(1) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 731 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

D) TOPOLOGY: linear

20

(11) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

9	TGGAATCCCT GGCCCCAITG GGCCTCCTGG TCCCCGTGGA CGATCAGGCG AAACCGGCCC	CGATCAG	TCCCCGTGGA	GGCCTCCTGG	GGCCCCATTG	TGGAATCCCT	
009	CCICICICIC TIGCAGGGIC CICCIGGCCC CGICGGICCC ICIGGCAAAG AIGGIGCIAA	TCTGGCA	cerceerccc	crccraaccc	TTGCAGGGTC	cereneres	5
540	CACCTACCCA GCCCCAGCGA CTCCCCAGCC TTCCCTGTGG TGACCACTCT TTCCTCACGA	TGACCAC	Trecereres	CTCCCCAGCC	GCCCCAGCGA	CACCTACCCA	c
480	GGTTCCATGA CTGAGCATGT GAAGAACTGG GGGGGAGTG GCTGGTGCTA TCAGGACAGC	GCTGGTC	GGGCGGAGTG	GAAGAACTGG	CTGAGCATGT	GGTTCCATGA	
420	GAGGGTAGGC AAGCCATGGA GCTATCCTGC TGGTTCTTGG CCAAATAGAG ACCAACTTAG	CCANATA	TGGTTCTTGG	GCTATCCTGC	AAGCCATGGA	GAGGGTAGGC	ın
360	acaaacatga atcagcctct cgctgtcaga cagaacagca ttttacaaag aggagcttag	TTTTAC	CAGAACAGCA	CGCTGTCAGA	ATCAGCCTCT	ACAAACATGA	
300	GGCAGGACTG GCTCATGTGC CTATGGCCAG AAAAGCGCCT GAGGCCACAA TGGCTGTAAG	GAGGCC	AAAAGCGCCT	CTATGGCCAG	GCTCATGTGC	GGCAGGACTG	.
240	AGGGAAATGC TGCTGCTTCT GGGGAAGCTG TGGGCTCAGG GGTCCTCACT CAGTAATGGG	GGTCCT	TGGGCTCAGG	GGGGAAGCTG	recreerrer	AGGGAAATGC	
180	ACATGGAGTT GGAAGATGGA GGGGCCCTT CAGAGAGTGT GGGCCTGTGT TCCCATGGGG	_ GGGCCT (CAGAGAGTGT	GGGGCCCIT	GGAAGATGGA	ACATGGAGTT	
120	GTCCTTCTGG AGACCAAGGT GCTTCTGGTC CTGCTGGTCC TTCTGGCCCT AGAGTAAGTG	TTCTGG	crecreerec	GCTTCTGGTC	AGACCAAGGT	greemen	LO.
09	AGAATATAGA TAGATATGTC TGTGCTGACC GTGGCCTTTT GCCTCTTCCT TCTACACAGG	GCCICI	GIGGCCTITI	TGTGCTGACC	TAGATATGTC	Agaatataga	

720	
CGGGAGGCTT	
CCCTACCATC	
GTCGAGGTGT	
CITCCCTGCT	
GTCCTGACTC	
TGCTGTAAGT	

GAGCTCTTT T

(2) INFORMATION FOR SEQ ID NO:16:

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(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

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(x1) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Cys

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(2) INFORMATION FOR SEQ ID NO:17:

SILLE
CHARACTE
SEQUENCE
(Ŧ)

(A) LENGTH: 5502 base pairs

TYPE: nucleic acid (B)

STRANDEDNESS: single

TOPOLOGY: linear <u>e</u> <u></u>

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "DNA"

(1x) FEATURE:

10

(A) NAME/KEY: CDS

(B) LOCATION: 1..5502

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG GAG AGC ACC TCC CCG CGA GGT CTC CGG TGC CCA CAG CTC TGC AGC Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser

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1260

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384	432	480	528	576	624
GGG AAC CCG GGA TGG CTC GCA GAG GCC GAG GCC AGG AGG CCA CCT CGA Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg 1365	CGG AGA AGC Arg Arg Ser 1395	CCT TCT GTC Pro Ser Val 1410	CGG	ACA	သသ
CCT	CGA GTC CAG CCA CCT GTC CAG ACT CGG AGA Arg Val Gln Pro Pro Val Gln Thr Arg Arg 1385	TCT GTC Ser Val	CCT CAG CGA CCC GCG GCT GCA CGG CGA GGG Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly 1420	TGC CCA GGA TGG ACA Cys Pro Gly Trp Thr 1440	CCT
Pro Pro	CGG	CCT	CGA (Arg (1425	CCA GGA Pro Gly 1440	CAG
AGG AGG Arg Arg 1375	CGA GTC CAG CCA CCT GTC CAG ACT Arg Val Gln Pro Pro Val Gln Thr 1385	GCA	CGG	CCA Pro 1440	TGT
AGG 1 Arg 1 1375	CAG Gln	GCT	GCA Ala	TGC Cys	GTG
GAG GCC Glu Ala	GTC Val (1390	CGG Arg	GCT	TGC	CCT GTG TGT
GAG Glu	CCT	GCC Ala	GCG	CAG Gln	AAA
GCA GAG GCC Ala Glu Ala 1370	Pro Pro	CAG CAG CAG ATA GCA GCC CGG GCT Gln Gln Gln Ile Ala Ala Arg Ala 1400	CCC Pro 11420	GGG AGA AAT GTC TGC GGG GGA CAG TGC Gly Arg Asn Val Cys Gly Gly Gln Cys 1430	ATC
GAG	GAG	ATA Ile	CGA Arg	GGG (Gly (1435	TGT
GCA (Ala (1370	GTC Val	CAG Gln	cag Gln	TGC	CAC
CTC	CGA (Arg 1385	CAG CAG Gln Gln 1400	CCT	GTC Val	AAC
TGG	CAG CTG CGT Gln Leu Arg	CAG Gln 1400	GAA ACC CCT CAG Glu Thr Pro Gln 1415	AAT Asn	ACC AAC CAC TGT ATC
GGA	CIG	GGC	CTG GAA ACC Leu Glu Thr 1415	GGG AGA AAT Gly Arg Asn 1430	AGC
AAC CCG Asn Pro 1365	cae Gln	CCC CGG Pro Arg	CTG	GGG G1y 1430	AAC
AAC Asn 1365	cag 31n	CCC Pro	CGC	ACT	TCA AAC AGC ACC AAC CAC TGT ATC AAA CCT GTG TGT CAG
GGG G1y	ACC Thr (1380	CAT	GCG	CTC .	ACA I
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672	720	768	816	864	912
TGC CGT Cys Arg 1475	GAG GAA Glu Glu 1490	GTG GAG AGA Val Glu Arg 1505	rg acc al Thr	CGG CGC Arg Arg	TCC AGG ACA Ser Arg Thr 1555
TGC ATC T	CCT GAG Pro Glu	TCA	GGA AGT CTA GTG Gly Ser Leu Val 1520	CCA ICT C Pro Ser A	CCG TCC A
CCC CAG GTC Pro Gln Val 1470	ATC	CGC	CCT CAC AGA AGC AGT GAG GCC AGA GGA AGT CTA GTG Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val 1515	CTG GTA CCA CCA TCA CCA CCT CCA TCT CGG CGC Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg 1530	CCC CTG CAG CAC TCA GGG CCG TCC AGG ACA Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr 1545 1550
GC AGG CC	CGC TGT GAG GAG GTC Arg Cys Glu Glu Val 1485	CCT GTG CCC AGA Pro Val Pro Arg 1500	AGT GAG GCC AGA Ser Glu Ala Arg 1515	CA CCA TCA ro Pro Ser	CCC CTG CAG CAG CAC Pro Leu Gln Gln His 1545
TCC TGC AGC Ser Cys Ser 1465	GGG GCG CGC TGT Gly Ala Arg Cys 1480	GCC AGG C Ala Arg P	AGA AGC A Arg Ser S	GTA CCA CCA Val Pro Pro 1530	CCC CTG C Pro Leu G 1545
CGA GGC	CGT	CAG AAT GCC Gln Aen Ala 1495	GGT CCT CAC Gly Pro His 1510	CCG CTG Pro Leu	TGG
TGT CAG AAC CGA GGC TCC TGC AGC AGG CCC CAG GTC TGC ATC TGC CGT Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg 1460 1465 1470	TCT GGC TTC Ser Gly Phe	TTT GAC CCT Phe Asp Pro	GCA CCC GGT Ala Pro Gly 3	AGA ATA CAG CCG Arg Ile Gln Pro 1525	CTC AGC CAG CCC Leu Ser Gln Pro 1540
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096	1008	1056	1104	1152	1200
GTT CGT CGG TAT CCG GCC ACT GGT GCC AAT GGC CAG CTG ATG TCC AAC Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn 1560 1570	GCT TTG CCT TCA GGA CTC GAG CTG AGA GAC AGC CCA CAG GCA GCA Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala 1575	CAT GTG AAC CAT CTC TCA CCC CCC TGG GGG CTG AAC CTC ACC GAG AAA His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys 1590	ATC AAG AAA ATC AAA GTC GTC TTC ACC CCC ACC ATC TGC AAG CAG ACC Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr 1605	TGT GCC CGG GGA CGC TGT GCC TGT GAG AAG GGT GAC ACC ACC Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr 1620	ACC TTG TAC AGT CAG GGT GGC CAT GGG CAT GAC CCC AAG TCT GGC TTC Thr Leu Tyr Ser Gln Gly His Gly His Asp Pro Lys Ser Gly Phe 1640
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CGT ATC TAT TTC TGC CAA ATC CCC TGC CTG AAT GGT GGC CGC TGC ATC Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile 1655 GGC CGG GAC GAG TGC TGG TGT CCA GCC AAC TCC ACA GGA AAG TTC TGC Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys 1670 CAT CTG CCT GTC CG CAG GAC GAC GAA CCT GCA GGG CGA GGT TCC His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser 1685 CGG CAC AGA ACC CTG CTG GAA GGT CCC CTG AAG CAA TCC ACC TTC ACG Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr 1700 TTG CTG CTT CTC TCT AAC CAG CTC GCC TGT AAG CAT TCC ACG TTC ACG Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr 1710 TTG CTG CTT CTC TCT AAC CAG CTC GCC TCT GTG AAG CAC TCG GTG AAG Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys 1730 GTG CAA ATT CAT CAC CCG CTC GAG GCC TCT GTG CAG GTG Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val 1775 1776 1776 1776 1776 1776 1776 1776 1777	1248	1296	1344	1392	1440	1488
GGT ATC TAT TTC TGC Arg Ile Tyr Phe Cys 1655 GGC CGG GAC GAG TGC Gly Arg Asp Glu Cys 1670 CAT CTG CCT GTC CCG His Leu Pro Val Pro 1685 CGG CAC AGA ACC CTG Arg His Arg Thr Leu 1700 CTG CCT CTC TCT AAC Leu Pro Leu Ser Asn 1720 GTG CAA ATT CAT CAC Val Gln Ile His His	TGC CTG AAT GGT GGC CGC TGC ATC Cys Leu Asn Gly Gly Arg Cys Ile 1660	TGC CyB	Ser	TTC ACG Phe Thr 1715	GTG AAG Val Lys 1730	CAC CAG GTG His Gln Val 1745
15 520	ATC TAT TTC TGC Ile Tyr Phe Cys 1655	ТСТ	CAT CTG CCT GTC CCG CAG His Leu Pro Val Pro Gln 1685	·	CTG CCT CTC Leu Pro Leu	CAA ATT CAT CAC CCG Gln Ile His His Pro 1735

1536	1584	1632	1680	1728	1776
G CTG GAG GAC AAC AGT GTG	CGC CCC CAC GGC AAC CTA GGC CAC AGC CCC Arg Pro His Gly Asn Leu Gly His Ser Pro 1770	ATA CCC GCT CGG GCC GGA GAG GCC CCT CGG CCA Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro 1785	TCT AGG CAT TAT GGA CTT CTG GGC CAG TGT TAC CTG Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu 1800	C CTA GGT AGT CTG ACT TCT o Leu Gly Ser Leu Thr Ser 1825	C TTC TGG GGG GTG ACC TCC r Phe Trp Gly Val Thr Ser 1840
CGG GTC CGG GGT GAG CTG GAC CCC GTG CTG Arg Val Arg Gly Glu Leu Asp Pro Val Leu 1750	CAT CGC CCC CAC GG His Arg Pro His Gl 1770	ATA CCC GCT CGG GCC Ile Pro Ala Arg Ala 1785	AGG CAT TAT GGA CTT CTG GGC Arg His Tyr Gly Leu Leu Gly 1805	CAG TGT GCT AAC CCC CTA GGT AGT Gln Cys Ala Asn Pro Leu Gly Ser 1820	GGC AGT GTG GGG ACC Gly Ser Val Gly Thr 1835
GCC CGG GTC CGG GGT C Ala Arg Val Arg Gly C 1750	GAG ACC AGA GCC TCT C Glu Thr Arg Ala Ser F 1765	TGG GCC AGC AAC AGC A Trp Ala Ser Asn Ser 1	CCA CCA GTG CTG TCT AGG Pro Pro Val Leu Ser Arg 1800	AGC ACG GTG AAT GGA CAG Ser Thr Val Asn Gly Gln 1815	CAG GAG GAC TGC TGT G Gln Glu Asp Cys Cys G
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1824			7817		1920			1968			2016				2064		-
ATT	ile	- (reu Feu	1875	AAG	Lys		AGG	Arg		AAG	Lys			GGT	Gly) ·
ST.	Val	3	ABn		IGC	Сув Lув	1890	TGC AGG	Сув		GAC	Asp			TCT		
4	Pro Val	. (CIG AAC CIC Leu Asn Leu		CIC	Leu		ACC	Thr Cys Arg	1905	IGG					31y (
GCT CCC TGC CCA CCC AGA CAA GAG GGT CCA GCC TTC CCA GTG ATT	Phe				TGC CTG ACC CTG GGC CTC TGC AAG	Gly Leu		TGC	Сув		TGC GTA TCG GAC AAG	Val Ser	1920		TCA CTG GGG	Ser Leu Gly Ser	
ָ ט ט	Ala Phe	1822	Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg		CTG	Leu		CTG	Leu Cys		TGC	CyB			TCA	Ser	1935
₹ U	Pro	į	ig ig	1870	ACC	Thr		TAC	17.		ည္ပ	Arg			SGG	Arg	
Į.	Gly	į	GGA Gly		CTG	Leu Thr	1885	TGC GTG AAC ACC AGG GGC AGC TAC	Ser		GAT CCG TCA AGG AGC CGC	Ser			CAG GGA CTA TGC TAC CGG	Gln Gly Leu Cys Tyr Arg	
GAG	Glu	;	g g		TGC	CyB		ညည	Gly Ser	1900	AGG	Arg			TGC	Сув	
A A D	Arg Gln		Pro		GAG	Glu		AGG	Arg		₹ 5	Ser Arg	1915		CIA	ren	
AGA	Arg	1650	75 E		AAT	Asn		ACC	Thr		ව්ට	Pro			GGA	Gly	1930
ט	Pro	e e	GAG Glu	1865	ATC	Ile	_	AAC	Aen Thr Arg	•	GAT	Asp			CAG	Gln	
S	Pro	Ę	Leu Glu Cys		TGC CAA GAT ATC AAT GAG	Asp Ile Asn	1880	GTG			CIG	Leu				Gln	
JOE	Сув		Gln		CAA	Gln		TGC	Cys Val	1895	GGC CTC ATG CTG				GTC TCC ATG CAG	Met	
CCC	Pro	7	Gly		TGC	Сув		GAG			CTC	Leu Met	1910		TCC	Ser	
GCT.	Ala		Gha Asn Gly		CAC	His		TCG GAG	Ser Glu		ညည	Gly			GTC	Val Ser	1925
TGT	Сув	6	Glu Glu	1860	AGC	Ser		GAC	Asp		ty S	Pro			GCT	Ala	
		٠ .	n			10				15				20			

ACC Thr 1940	ACC TGC ACC CTG CCT TTG GTT CAT CGG ATC ACC AAG CAG ATA TGC TGC THr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys 1940 1945 1950	ACC	CTG	CCT	TTG Leu 1945	CCT TTG GTT CAT Pro Leu Val His 1945	CAT	CGG	CGG ATC ACC AAG Arg Ile Thr Lys 1950	ACC Thr 1950	AAG Lys	CAG Gln	ATA Ile	TGC	TGC Cys 1955	2112	
TGC	TGC AGC Cys Ser	CGT	GTG	GGC 7 G1Y 1 1960	AAA Lys	GGC AAA GCC Gly Lys Ala 1960	GTG GGC AAA GCC TGG Val Gly Lys Ala Trp 1960	GGT Gly	TGG GGT AGC ACA TGT GAA CAG Trp Gly Ser Thr Cys Glu Gln 1965	ACA Thr	TGT Cys	gaa Glu	cag gln	TGT Cys 197(CCC	2160	
CTG			GGC ACA GAA GCC TTC AGG Gly Thr Glu Ala Phe Arg 1975	GAA Glu	GCC	TTC	AGG	GAG 7 Glu 1980	GAG ATC Glu Ile 1980	TGC	CCT	GCT	GGC G1Y 1985	GGC CAT Gly His 1985	GGC	2208	
TAC	ACC		TAC TCG AGC Tyr Ser Ser 1990	AGC	JCA	gac Asp	ATC (Ile / 1995	CGC Arg	TAC TCG AGC TCA GAC ATC CGC CTG TCT ATG AGG AAA GCC GAA Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu 1990	rcr	ATG Met	AGG Arg 2000	AAA Lys	GCC	GAA	2256	
GAA	GAA GAG GAA CTG GCT AGC CCC TTA AGG GAG CAG ACA GAG CAG AGC ACT Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr 2005	GAA Glu	CTG	GCT Ala	AGC	CCC 7	CCC TTA AGG GAG Pro Leu Arg Glu 2010	AGG	GAG Glu	CAG Gln	ACA (Thr (ACA GAG Thr Glu 2015	CAG	AGC	ACT Thr	2304	
GCA Ala 2020	GCA CCC Ala Pro 2020	CCC CCA CCT GGG CAA GCA GAG AGG CAA CCA CTC CGG GCA GCC ACC Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr 2035	CCT	GGG G1y	CAA (Gln 1 2025	GCA	GAG	AGG Arg	CCA CCT GGG CAA GCA GAG CAA CCA CTC CGG GCA GCC ACC Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr 2025 2030	CCA (Pro] 2030	CTC	CGG Arg	SCA Ala	GCC	ACC Thr 2035	2352	

2400	2448	2496	2544	2592	2640
GCC ACC TGG ATT GAG GCT GAG ACC CTC CCT GAC AAA GGT GAC TCT CGG Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg 2040	GCT GIT CAG ATC ACA ACC AGT GCT CCC CAC CTA CCT GCC CGG GTA CCA Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro 2055	GGG GAT GCC ACT GGA AGA CCA GCA CCA TCC TTG CCT GGA CAG GGC ATT Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile 2070	CCA GAG AGT CCA GCA GAA GAG CAA GTG ATT CCC TCC AGT GAT GTC TTG Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser ABP Val Leu 2085	GTG ACA CAC AGC CCA GAC TTT GAT CCA TGT TTT GCT GGA GCC TCC Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser 2100	AAC AIC TGT GGC CCT GGG ACC TGT GTG AGC CTC CCA AAT GGA TAC AGA Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
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2688	2736	2784	2832	2880	2928
TGT Cys	GGG CGC Gly Arg	GGC TAC Gly Tyr	GAT GAG Asp Glu 2195	GAG Glu	лал Гув
TAC	5 GGG G1y	GGC	gat Abd	ACG GAG Thr Glu 2210	AGG
CAA GAC TAC Gln ASP TYT	2145 AGA (CCT	ATC	AAC	GTC // Val // 2225
Gla Gla	2145 GAA GGA AGA Glu Gly Arg 2160	TAT Tyr	GAT Asp	AGC	ATG Met
AGC	GAA	TGC ' Cys ' 2175	CAA Gln	TGC	ATC ATG GTC AGG AAA Ile Met Val Arg Lys 2225
CCC	TGT Cys	CTC	TGC CAA GAT Cys Gln Asp 2190	CGA	TAC
CAC	2140 AGG AAC CCC TGT Arg Asn Pro Cys 2155	GGC TCC TAC TCC TGC CTC Gly Ser Tyr Ser Cys Leu 2170	GAG Glu	TGC AGT GGT GGG CGA TGC AGC AAC ACG GAG Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu 2205	96C
CTA	2140 AAC (ABB)	TCC	CAG GAG Oln Olu	GGT Gly	CGG (Arg (
CAG Gln	AGG / Arg /	TAC	ACA Thr	AGT Ser	GAT
	ATG Met	TCC Ser 32170	gac Asp	ТСУВ	TGT
GGC Gly	TGT Cys	GGC TCC TAC TCC TGC Gly Ser Tyr Ser Cy8 2170	GGA GAC ACA Gly Asp Thr 2185	grg Val	gAG 31u
ro Fro	3AG 31u		CTC	CAG CCC GGG GTG Gln Pro Gly Val 2200	TAC CAC TGC GAG TGT GAT CGG GGC TAC Tyr His Cys Glu Cys Asp Arg Gly Tyr 2215
AGC	2135 AAC (Asn (GTC AAC AGT GTG Val Asn Ser Val 2165	ACC	Pro	CAC ; His (
тсс Сув	GAC ASP ASP 2150	AAC Asn	GTC Val	cag Gln	TAC
GTC	GAT	GTC AAC Val Asn 2165	CTA	GAG Glu	TCG
CyB	ACT	TGT	ACA CTA GTC ACC CTC GGA GAC ACA CAG GAG TGC CAA GAT ATC GAT GAG Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu 2180	ТЭТ	GGC
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2976	3024	3072	3120	3168	3216
TGT CAA GAT ATC AAC GAA TGC CGT CAC CCT GGT ACC TGC CCT Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro 2230	CTG GCC TGT Leu Ala Cys	GAG GAG GGC TAT GTA GGC CAG AGT GGG AGC TGT GTA GAT GTC AAT GAG Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu 2260	ACC CCT GGG ATA TGT ACC CAT GGA AGG TGC ATC AAC ATG GAA Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu 2280	TGT GAG CCG GGC TAT GAG GTC ACC CCA GAC Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp 2300	GCC AGC CGA GCC TCG TGC Ala Ser Arg Ala Ser Cys 2320
ТЭС	600 A13	AAT	ATG (Met (2290	20 CT	TCG
ACC	CTG GCC Leu Ala	GTC AAT Val Aen	AAC Abn	ACC Thr 2305	GCC
GGT ACC Gly Thr 2240	CyB	gat Asp	ATC Ile	GTC ACC CCA Val Thr Pro 2305	CGA GCC Arg Ala 2320
CCT	ACT Thr (2255	GTA Val	TGC	GAG	AGC Ser 2
CAC	TAC	TGT GTA GAT Cys Val Asp 2270	AGG	TAT	SCC 2
GAA TGC CGT Glu Cy8 Arg 2235	GGG AGA TGC GTC AAC TCC CCT GGC TCC TAC ACT TGT Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys 2245	AGC	GGA 1 Gly 1 2285	66C 61Y	GGC TGC CGA GAT GTG GAC GAG TGT GCC AGC Gly Cys Arg Asp Val Asp Glu Cys Ala Ser 2310
TGC CyB	GGC G1y	666 617	CAT	CCG GGC Pro Gly 2300	GAG '
GAA 'Glu (2235	CCT Pro	AGT	ACC	GAG	GAC (Asp (2315
AAC	TCC Ser 2250	CAG Gln	тст	TGT	GTG
ATC	AAC	GGC CAG AGT Gly Gln Ser 2265	ATA	Ser	CGA GAT GTG GAC GAG TGT Arg Asp Val Asp Glu Cys 2315
GAT Asp	GTC Val	GTA Val	GGG ATA Gly Ile 2280		Arg 1
rgr caa Cys Gln 2230	ТСС	TAT	CCT	AGA TGC Arg CyB 2295	TGC CYB 7
TGT Cys 2230	AGA Arg	GGC G1y	ACC	TTT Phe	AAG GGC TGC Lys Gly Cys 2310
CAC	GGG ; Gly ; 2245	GAG Glu	CTG	Ser	AAG (Lys (
GGA Gly	GAT	GAG GAG Glu Glu 2260	TGT CTG Cys Leu	аас 1 а1у 8	AAG 1
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3264	3312	3360	3408	3456	3504
CCC ACG GGC CTC TGC CTC AAC ACG GAG GGC TCC TTC ACC TGC TCA GCC Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala 2325	GGC ACT GCC TGT GAA GAC Gly Thr Ala Cys Glu Asp 2350	GCC TTC CCT GGA GTC TGC CCC ACA GGC GTC TGC ACC Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr 2360 2365	GTA GGC TCC TTC TCC TGC AAG GAC TGT GAC CAG GGC TAC CGG Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg 2375	gaa gat gat gag tgt gaa ggt 31u aep Val aep glu Cye glu gly 2400	TGC CGG GGA GGC GAA TGC AAG AAC ACA GAA GGT TCC Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser 2410
CCC ACG GGC CTC TGC CTC AAC ACG Pro Thr Gly Leu Cys Leu Asn Thr 2325	TGT CAG AGC GGG TAC TGG GTG AAC GAA GAT Cye Gln Ser Gly Tyr Trp Val Aen Glu Aep 2340	TTG GAT GAA TGT GCC TTC CCT GGA Leu Asp Glu Cys Ala Phe Pro Gly 2360	AAT ACT GTA GGC TCC TTC TCC TGC Asn Thr Val Gly Ser Phe Ser Cys 2375	CCC AAC CCC CTG GGC AAC AGA TGC (Pro Asn Pro Leu Gly Asn Arg Cys (2395	CCC CAA AGC AGC TGC CGG GGA GGC Pro Gln Ser Ser Cys Arg Gly Gly G2405
	ហ	10	15	20	

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3552	3600	3648	3696	3744	3792
ATG Met 2435	CAC	CCC	GAA	GAG Glu	CCA Pro 2515
Acc	CCT CAC Pro His	GCA (Ala)	JAT (ACA C	
GGC GIY	GCT	TGT (Cys 1 2465	STT (Mac A	770 J
TAC CAA TGC CTC TGT CAC CAG GGC TTC CAG CTG GTC AAT GGC ACC ATG Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met 2420 2430	TGT	CTC	TGC CAG GAT GTT GAT Cys Gln Asp Val Asp 2480	GCA GCC ACA GAC CCG TGT CCG GGA GGA CAC TGT GTC AAC ACA GAG Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu 2485	GCT TCC TTC CAG CCC TCC Ala Ser Phe Gln Pro Ser 2510
GTC	AAT GAG TGT GTT GGG GAA GAG CAT TGT ABN Glu Cys Val Gly Glu Glu His Cys 2440	TGC	CAG (Glu	TGT (Cys 7 2495	rrc o
CTG Leu 2430	GAG	TTC	TGC	CAC His	TCC 7 Ser 1 2510
CAC CAG GGC TTC CAG His Gln Gly Phe Gln 2425	TGT GAG GAC GTG AAT GAG TGT GTT GGG GAA GAG CAT Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His 2440	CTC AAC AGC CTG GGC TCC TTC TTC Leu Asn Ser Leu Gly Ser Phe Phe 2455	GCT AGT GCT GAG GGG GGC ACC AGA TGC Ala Ser Ala Glu Gly Gly Thr Arg Cys 2470	GGA	GCT Ala
TTC	666 61y	TCC TTC Ser Phe 2460	GCT GAG GGG GGC ACC AGA Ala Glu Gly Gly Thr Arg 2475	gga Gly	TCC TTC AGC TGT CTG TGT GAG ACT GCT Ser Phe Ser Cys Leu Cys Glu Thr Ala 2505
GGC	GTT Val	CTG GGC Leu Gly	GGC G1y 2475	TGT CCG GGA Cys Pro Gly 2490	TGT CTG TGT GAG ACT Cys Leu Cys Glu Thr 2505
CAG Gln	TGT Cys	CTG	GGG G1y	TGT Cys 2490	Сув
CAC (His (2425	GAG Glu	AGC	GAG	CCG	CTG Leu 2505
TGT	AAT (Asn (2440	CTC AAC Leu Asn 2455	GCT Ala	GAC CCG Asp Pro	TGT
CTC	GTG	CTC Leu 2455	AGT	AGA	AGC
TGC	GAG GAC Glu Aep	TGC	GCT AGT Ala Ser 2470	GCC	TTC
TAC CAA Tyr Gln 2420	GAG Glu	GAG Glu	TTT	GCA (TCC Ser
TAC Tyr 2420	ТЭТ	66C	GGC TIT	TGT GCA GCC ACA Cys Ala Ala Thr 2485	GGC TCC Gly Ser 2500
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	2600 2605 2610	
·,	Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu	٠
4080	GGC TIC GAG ACC TCA CCA TCA GGC TGG GAG TGT GTT GAT GTG AAC GAG	
		20
	2580 2585 2590 2595	
	Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln	
4032	GGC TTC TGT GAC AAC ACG GAC GGC TCC TTC CGC TGC CTG TGT GAC CAG	
	2565 2570 2575	15
	Cys lle Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His	
3984	TGC AIT GAC AIA GAI GAA IGI GCC AAI GAC ACI GIG IGI GGG AAC CAI	
*	2550 2555 2560	
	Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp	10
3936	TGC ATC CTG GAC TGC CAG CCT GGA TTC TAT GTG GCG CCA AAT GGA GAC	
	2535 2540 2545	
	Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg	
3888	CCG GTG TGC GGA GCC TGG AGG TGT GAG AAC AGT CCT GGT TCC TAC CGC	Ŋ
	2520 2525 2530	
	Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp	
3840	GAC AGC GGA GAA TGT TTG GAT ATT GAT GAG TGT GAG GAC CGT GAA GAC	

4128	4176	4224	4272	4320	4368
TGT GAG CTC ATG ATG GCA GTG TGT GGG GAT GCG CTC TGT GAG AAC GTG Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val 2615 2620	GAA GGC TCC TTC CTG TGC CTT TGC GCC AGT GAC CTT GAG GAG TAC GAC Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp 2630	GCA GAA GAA GGA CAC TGC CGT CCT CGG GTG GCT GGA GCT CAG AGA ATC Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile 2645	CCA GAG GTC CGG ACA GAG GAC CAG GCT CCA AGC CTT ATC CGC ATG GAA Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu 2660 2660	TGC TAC TCT GAA CAC AAT GGT GGT CCT CCC TGC TCT CAA ATC CTG GGC Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly 2680	CAG AAC TCC ACA CAG GCC GAG TGC TGC ACT CAG GGT GCC AGA TGG Gln Asn Ser Thr Gln Ala Glu Cys Cys Thr Gln Gly Ala Arg Trp 2695
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Cys Ala Pro Cys Pro Ser Glu Asp Ser 2715 CCC AGT GGT CAA GGT TAC ATC CCA GTG Pro Ser Gly Gln Gly Tyr Ile Pro Val 2730 CAA ACC ATG TAT ACA GAT GCC GAT GAA Gln Thr Met Tyr Thr Asp Ala Asp Glu 2745 CTC TGC CAG AAT GGC CGA TGC TCA AAC Leu Cys Gln Asn Gly Arg Cys Ser Asn 2760 CTG TGC AAC CCT GGC TAC CAC TAT GAT Leu Cys Asn Pro Gly Tyr His Tyr Asp 2775 CTG TGC AAC CCT GGC TAC CAC TAT GAT GAT GCT TGC AAC GAT GCT CAC TAT GAT Asp 2760 CTG TGC AAC CCT GGC TAC CAC TAT GAT GAT TGC CAG GAT TGC CAG GAC TTG GCC ABB ABD CAT TGC CAG GAC TTG GCC ABD TATS ABD LEU ALA ABD TATS ABD LEU ALA ABD TATS CAG GAT TGC CAG GAT TGG CAG GAT TGG CAG GAT TGG CCC ABD TGC CAG GAC TTG GCC CAG GAT TGG CAG TAG GAT TGG CAG TGG TAG TGG TAG GAT TGG CAG TAG GAT TGG CAG TGG TAG TGG TAG TGG TAG GAT TGG CAG TGG TAG GAT TGG CAG TGG GAT TGG CAG TGG GAT TGG CAG TGG TGG TGG TGG TGG TGG TGG TGG TGG T					*	
Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe 2710 2715 CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala 2725 TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu 2745 CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro 2760 ATT TGC CTG TGC AAC CTC GGC TAC CAC TAT GAT GCC TCC ATT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Ile Cys Leu Cys Asn TGC CAG GAC TGC TGT GAG AAC CCG GAT GAC TAC GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TGT GAC TGT GAG AAC CCG GAT CAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAG GAT CAC GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAC CAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAC CAG GAA TGC CAG GAC TTG GCC TGT GAG AAC CCG GAT GAC CAG GAT TGC CAG GAC TGT GAG AAC CCG GAT GAC GAA TGC CAG GAC TGT GAG GAC TGT GAG AAC CCG GAT GAC GAA TGC CAG GAC TGT GAG AAC CCG GAT GAC GAG GAC TGC TGT GAG AAC CCG GAT GAC GAG GAC TGC TGT GAG AAC CCG GAT GAC GAG GAC TGC TGT GAG AAC CCG GAT GAC GAG GAC TGC TGT GAG AAC CCG GAT GAC GAG GAC TGC GAG GAC TGT GAG AAC CCG GAT GAC GAG GAC TGT GAG GAC TGT GAG AAC CCT GAG GAT GAC GAG GAC TGT GAG AAC GAG GAC TGT GAG GAC TGT GAG AAC GAG GAC TGT GAG AAC GAG GAC TGT GAG AAC GAG G	4416	4464	4512	4560	4608	4656
GGI GIJ GIJ Z74 Z74 Z74 Z74 Z74 Z74 Z74 Z74 Z74 Z74	AAG GCC TGT GCG CCC TGC CCA TCT GAG GAC TCA GTT GAA TTC Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe 2710 2715	CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC TGG Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp 2725	TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu 2745	CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT GGC Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly 2760	AIT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC AGG Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg 2775 2785	TGC CAG GAT CAC AAC GAA TGC CAG GAC TTG GCC TGT GAG AAC GGT Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly 2790
15 10 20 20	9. G.					•

4704	4752	4800	4848	4896	4944
CAT TGC CTC TGC AAT CCC CCC His Cys Leu Cys Asn Pro Pro 2815	TGT GTG AAC ACG ACC AGC Cys Val Asn Thr Thr Ser 2830	GAC ATC CAC ATG GAC ATC TGC TGG AAA Asp Ile His Met Asp Ile Cys Trp Lys 2845	ACC AAT GAT GTG TGC AGC CAG CCC TTG CGT GGG CAC CAT ACC Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr 2855	G GAG GCC TGG AGC CAG CAA Y Glu Ala Trp Ser Gln Gln 2880	GAG GTC TAC GCT CAG CTG TGC Glu Val Tyr Ala Gln Leu Cys 2895
GGC TCC TTC Gly Ser Phe 2810	AGT GGG CAG CGC TGT Ser Gly Gln Arg Cys 2825		TGC AGC CAG CCC TTG Cys Ser Gln Pro Leu 2860	TGC TGC CAA GAT GGG GAG GCC Cys Cys Gln Asp Gly Glu Ala 2875	AGG AGC TCT GA Arg Ser Ser Gl 2890
TGT GTG AAC CAA GAA Cys Val Asn Gln Glu 2805	CTA GAC CTC Leu Asp Leu	G GAC TTC CCT GAC CAT u Asp Phe Pro Asp His 2840		ACA GAA IGC Thr Glu Cys 2870	crd rgc ccg ccc Leu Cys Pro Pro
GAG TG Glu Cy 28	5 CTC ACC Leu Thr 2820	ACG GAG GAC 10 Thr Glu Asp	AAA GTC Lys Val 15	ACC TAT Thr Tyr	TGC

4992	5040	2088	5136	5184	5232
ATC CAC TTC CGG :Ile His Phe Arg 2915	CCT GAA AAC CTC Pro Glu Asn Leu 2930	GGC CCC GAG GAC Gly Pro Glu Asp 2945	TCC AAC CCA GCC AGC CAG CCG GGA GAC Ser Asn Pro Ala Ser Gln Pro Gly Asp 2955	GAA CTT CAG CCT Glu Leu Gln Pro	TTC GAA GGC CTT Phe Glu Gly Leu 2995
GGC GGA GCA GGG ATC Arg Gly Ala Gly Ile 2910	TG GAC GAT CTG eu Asp Asp Leu 2925	TAT AAC TAC CTA GGC CCC GAG Tyr Aen Tyr Leu Gly Pro Glu 2940	TCC AAC CCA GCC AGC CAG CCG Ser Asn Pro Ala Ser Gln Pro 2955	TG CAG CCC TCT GAA CTT eu Gln Pro Ser Glu Leu 2975	CC CCT GCC TCC ro Pro Ala Ser 2990
CGG AIT GAG GCA GAG CGC GGA GCA GGG AIC CAC ITC CGG Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg 2905	TAT GAG TAT GGC CCT GGC CTG GAC GAT CTG CCT GAA AAC CTC Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu 2920	CCA GAT GGG GCT CCC TTC T Pro Asp Gly Ala Pro Phe T 2935	GCC CCT GAG CCT CCC TTC TCC A Ala Pro Glu Pro Pro Phe Ser A 2955	AAC ACA CCT GTC CTT GAG CCT CCT CTG CAG CCC TCT GAA CTT Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu 2965 2975	CAC TAT CTA GCC AGC CAC TCA GAA CCC CCT GCC TCC TTC GAA GGC CTT His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu 2980
AAC GTG GCT C Aen Val Ala A 2900	5 CCA GGC TAT G Pro Gly Tyr G	TAC GGC Tyr Gly	ACT		CAC His 2980
	_ .	10	5	20	

.GC 5280 .ya	CAG 5328 Gln	AC 5376 Bp	ACA 5424 Thr	CCA 5472 Pro 3075	5502
GGC CGC TGC Gly Arg Cy8 3010	GAG GGC TTC CAG Glu Gly Phe Gln 3025	TGT GAA GAC Cys Glu Asp	GAG AAC I	GAG Glu	
GAG AAT GGC Glu Aen Gly	TTT Phe	AAC GAG TGT Asn Glu Cys 3040	CAC GGT CAC TGT GAG AAC His Gly His Cys Glu Asn 3055	GGT TAC GTG GCA Gly Tyr Val Ala 3070	
CTG AAT GGC TGT Leu Asn Gly Cys 3005	GAC TGC Asp Cys	TGT GTG GAT GTG AAC Cys Val Asp Val Asn 3035	CAC GGT	CCA Pro	TAG
c CTG AAI e Leu Asn	CGG GAG GGC TAC ACT TGC GAC TGC Arg Glu Gly Tyr Thr Cys Asp Cys 3015		c rgr gca u Cys Ala 50	TGC CAC TGT TCG Cys His Cys Ser 3065	TGT GCG GCC AAG GAG TAG
TGT GGC ATC Cys Gly Ile 3000	CGT GTG CGG GAG GGC TAC Arg Val Arg Glu Gly Tyr 3015	ACA TTG GCC Thr Leu Ala	CA CGA CTC la Arg Leu 3050	CGC TGC CAC Arg Cys His 3065	F GCG GC
GAG GAA TO Glu Glu Cy 30	GTG CGG G9 Val Arg GJ 3015	GCG CCC AC Ala Pro Th	GG CCT GK ly Pro Al	TCC TAT CO Ser Tyr At	CCA CAC TG
CAG GCT GAG GAA TGT GGC ATC CTG AAT GGC TGT GAG AAT GGC CGC TGC Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys 3000	GTG CGT G	CTG GAT G(Leu Asp A)	TTG AAC GGG CCT GCA CGA CTC TGT GCA CAC GGT CAC TGT GAG AAC ACA Leu Aen Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Aen Thr 3045	GRG GGT TC Glu Gly Se 3060	න නන නන
	ru O	10 1	1 I		20 GGC CCC

(2) INFORMATION FOR SEQ ID NO:18:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1834 amino acids

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(B) TYPE: amino acid

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:18:

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Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser

His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys

Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val 20 Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu

H18 80	Asp	Gln	Arg	Ser	Val 160	Arg	Thr
Ser	Pro 95	Ala	Pro	Arg	Ser	G1y 175	
Leu Trp His Pro Val Gly 75	Ser Leu Phe Arg Glu Pro Asp 90	Gln Pro Ala 110	Glu Ala Arg Arg Pro 125	Arg	Pro	Ala Ala Ala Arg Arg Gly Arg 170	Pro Gly Trp 190
Val	Arg	Gln	Arg 125	Thr	Ala	Arg	Pro
Pro	Phe	Asn	Arg	Gln Thr 140	Ile Ala Ala Arg Ala Ala Pro 155	Ala	
H18 75	Leu	Trp	Ala	Val	Arg 155	Ala	Gly Gly Gln Cye Cye 185
ĮĮ.	Ser 90	Glu	glu	Pro Val	Ala	Ala 170	gln (
Leu	Tyr	Ser 105	Ala	Pro	Ala	Pro	Gly (185
Arg	Lys val	Pro	Glu Ala 120	Gl n	Ile	Arg	317
Asn	L γв	Ser	Ala	Val 135	Gln	Gln	Cys
Arg Asp Ala Asn Arg 70	Ala Ala 85		Leu Ala	Arg	Gln Gln Gln 150	Pro Gln Arg	val
Asp	Ala 85	Gly Leu	Trp	Arg	Gln	Thr 165	Asn
Arg	Ala	Pro 100		Gln Gln Leu Arg Arg Val Gln 130	ыу	Glu	Arg 180
Ser	Ala	Val	Pro Gly 115	Gln	Arg	Leu	Gly
Pro Ala 65	Pro Ala	Pro Val	Asn	Gln 130	Pro	Arg	Thr (
Pro 65	Pro	Ala	Gly	Thr	Hi8 145	Ala	Leu
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Pro	Cys Arg	Glu 240	Arg	Thr	Arg	Thr	Asn
Pro Pro		Glu	Glu Arg 255		Arg Arg	Arg	Ser Asn
Gln	11e	Glu	Ser Val	Leu Val 270		Ser	
Сув 205	С у в	Pro		Ser	Pro Ser 285	Pro	Leu
Val	Val 220	Ile	Arg	Gly	Pro	Gly Pro 300	Asn Gly Gln Leu Met 315
Pro	Gln	Glu Glu Val 235	Pro Arg 250	Arg	Pro	Ser	Gly
Lyв	Pro	Glu	Pro 250	Glu Ala 265	Ser	His	Aen
Ile .	Arg	G1 u	Val		Pro	Gln	Ala
Cy8 200	Ser	Cya	Pro	Ser	Pro 280	Gln	Gly Ala
нів	Сув 215	Gly Ala Arg 230	Pro Gln Asn Ala Arg 245	Ser	Pro	Leu 295	Thr
Asn	Ser	Ala 230	Ala	Arg	Val	Pro	Ala 310
Thr	Gly	Gly	Asn 245	His	Leu	Trp	Pro Ala 310
Asn Ser 195	Arg	Phe Arg	Gln	Pro 260	Pro	Pro	Tyr
Asn 195	Gln Asn Arg 210			Gly	Gln Pro Leu 275	Gln	
Ser		Gly	Asp	Ala Pro Gly Pro His 260	ile	Ser 290	Arg
Thr	Сув	Ser 225	Phe	Ala	Arg	Leu	Val Arg Arg 305
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Ala	Гув	Thr	Thr	Phe 400	11e	Сув	Ser
Ala 335	Glu			Gly Phe 400	Cy8 415		Gly (
Gln	Thr 350	Lys Gln	Asp	Ser	Arg	Ly8 430	Arg
Pro	Leu	Су <i>в</i> 365	Gly	Lys Ser	Gly	Gly	Gly .
Ser Pro	Leu Asn Leu	11e	Glu Lys Gly Asp Thr 380	Pro	Pro Cys Leu Asn Gly Gly Arg 410	Pro Ala Asn Ser Thr Gly Lys Phe 425	Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg 440
	Leu	Thr	Glu	Asp 395	Asn	Ser	Pro ;
Авр 330		Pro	Cys	His	Leu 410	Asn	3lu
Gly Leu Glu Leu Arg Asp Ser 325	Trp Gly 345		Ser	Ser Gln Gly Gly His Gly His Asp Pro 390	Сув	Ala .	Arg (
Leu	Pro	Phe 360	Asn	His	Pro	Pro	Asp 440
Glu	Pro	Val	Ala 375	Gly	Ile	Сув	Pro
Leu	Ser	Val	Сув	G1y 390	Gln	Trp	Gln
Gly 325	Leu	Lys val val Phe Thr 360	Arg	Gln	Phe Cys Gln 405	Сув	Pro
Ser	Asn His Leu 340		Gly	Ser	Phe	Arg Asp Glu Cys 420	
Pro	Asn	Lys Ile 355	Arg		Tyr	Авр	Pro Val 435
Ala Leu		Lув	Ala 370	Leu Tyr	Ile	Arg	Lea
Ala	Hie Val	Ile	Cys Ala Arg Gly Arg Cys Ala Asn 370	Thr 385	Arg	Gly	His 1
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Thr	Lys 480	Val	Val	Pro	Pro	Leu 560	Ser
Phe	Val	Gln 495	Ser	Ser	Arg	Tyr]	
Thr		His		нів	Pro	Сув	Leu Thr
	Ser Leu	Ile	Авр	Gly 525		Gln (Ser 1
Gln Ser 460	Asn Pro 475	Gln	Glu	Leu	Glu Ala 540	Gly	aly (
Thr Leu Leu Glu Gly Pro Leu Lys 455	Asn 475	Val	Pro Val Leu Glu Asp Asn 505	Gly Asn Leu	вιу	Leu 555	
Leu	Val	Ser 490	Val	βlγ	Ala	ne T	Asn Pro Leu
Pro	Ser	Glu Ala		нів	Arg	Gly Leu	Asn 1
βlγ	Ala	Glu	Leu Asp	Pro 520	Ala	Tyr	Ala
Glu 455	Gln Leu 470	Pro	Leu	Arg	Pro Ala 535	His	Сув
Leu	Gln 470	Pro	Glu	нів	Ile	Arg 550	Gln
Leu	Aen	His 485	Gly	Ser	Ser.	Ser	Asn Gly Gln 565
Thr	Ser	His	Arg 500	Ala	Asn	Leu	Asn
His Arg 450	Leu	Ile	Val	Arg 515	Ser	Val	Val
	Pro	Gln	Arg	Thr	Ala 530	Pro	Thr
Arg	Leu 465	Val	Ala	Glu	ĘĮ.	Pro 545	Ser
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Ser	Ile	ren	Lys 640	Arg	LyB	олу	Cys
Thr	Val	Leu Asn	Сув. Lys 640	Сув 655	ABp	Ser	Сув Сув
Val 590	Pro	Leu	Leu	Thr	Ser 670		Ile
Trp Gly	Phe 605	Lув Arg 620	Gly		Val	Leu Gly 685	Gln
Trp	Gln Glu Gly Pro Ala 600		Leu Thr Leu Gly Leu 635	Leu Cys	ζ	Ser	Ly8 700
Phe	Pro	T	Thr 635	77	Arg	Arg	Thr
Thr	Gly	Gly	Leu	Ser 650	Ser	17.	Ile
Ser Val Gly Thr Phe 585	Glu	Сув Pro Gln Gly Туr 615	Сув	Thr Arg Gly Ser Tyr 650	Arg Ser 665	Сув	Arg
Val	Gln 600	Pro	Ile Asn Glu 630	Arg	Ser	Leu Cys 680	
Ser	Pro Pro Arg		Asn	Thr	Pro	Gly	Leu Val His 695
Gly	Pro	Glu	Ile 630	Val Agn 645	Asp	Gln Gly	Leu
	Pro	Leu	Asp	Val 645	Leu	Gln	Pro
Сув 580	Pro Cys 595	Asn Gly Gln Leu Glu 610	Gln Asp	Cya	Met Leu Asp 660	Met	Leu
Asp		Gly	Сув	gJu	Leu	Ser 675	Thr
Gln Glu Asp Cys Cys 580	Cys Ala	Asn 610	His	Ser Glu	Pro Gly Leu	Val	Cys Thr 690
Gln	Сув	Glu	Ser 625	Asp	Pro	Ala	Thr
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Pro 720	Gly	Glu	Thr	Thr	Arg 800	Pro	Ile
Thr Cys Glu Gln Cys 715	H18 735	Ala	Ser	Ala	Ser	Val 1 815	
Gln	Gly	ьув 750		Ala	Asp	Prg 1	Gly Gln Gly 830
Glu	Pro Ala	Arrg	Glu Gln 765	Arg	Gly	Ala i	3ly C
Cys	Pro	Met	Thr	Leu Arg 780	Pro Asp Lys Gly Asp 795	Pro Ala Arg	Pro (
Thr 715	Сув	Ser	Gln	Gln Ala Glu Arg Gln Pro 775	Авр 795	ren	Leu Pro
Ser	11e 730	Arg Leu 745	Glu	Gln	Pro	His 810	Ser]
Gly	Glu	Arg 745	Leu Arg 760	Arg	Leu	Pro	Pro (825
Trp	Arg	Ile	Leu 760	Glu	Thr	Ala	Ala 1
Lys Ala 710	Phe	Asp	Pro	Ala 775	Glu Ala Glu Thr 790	Ser	Pro
. Lyв 710	Glu Ala 725	Ser	Ser	Gln	Ala 790	Thr	Arg
Gly	Glu 725	Ser	Ala	Gly	Glu	Thr 805	Gly
val	Thr	Ser 740	Glu Leu Ala Ser 755	Pro	Ile	Ile	Thr 820
Arg	Pro Gly	Tyr	Glu 755	Pro	Trp	Gln	
Ser		Thr	Glu	Pro 770	Thr	Val	Asp
Сув 705	Leu	Tyr	Glu	Ala	Ala 785	Ala	Gly Asp Ala
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Leu	Ser	Arg 880	Cys	Arg	Tyr	g]u	glu
Val	Ala	Tyr	Tyr 895	Asn Pro Cys Glu Gly Arg Gly Arg 905	Gly	Авр (Thr. C
Ser Asp 845	Gly		Авр	Arg 910	Pro	Ile	Asn '
Ser 845	Ala	Pro Asn Gly	Gln Asp	Gly	Tyr Pro 925	Авр	Ser 1
Ser	Phe 860	Pro	Ser	Glu	Сув	Gln Asp Ile Asp 940	Ser Gly Gly Arg Cys Ser Asn Thr
Pro	Сув	Leu 875	Pro	Сув	Leu	Сув	Arg (
Ile	Pro	Ser	Gln Leu His 890	Pro	Cys		Gly
Val	Asp	Val	Leu	Asn 905	Ser	Gln Glu	Gly (
Glu Glu Gln Val 840	Asp Phe 855	CyB	Gln	Arg	TYT 920	Thr	Ser
Glu	Авр 855	Thr.	Tyr	Met Arg	Ser	Asp 935	Сув
	Pro Pro	Pro Gly Thr 870	Pro Gly Tyr 885	CyB	Gly	Gly	Val (
Ala		Pro	Pro 885	Glu	Val	Leu Gly	Gly Val
Ser Pro Ala 835	Ser	$_{ m Gl\gamma}$	Ser	Asn Glu 900	Ser	Thr	Pro (
	His	Сув	Cya	Asp	Asn Ser 915		Gln
Glu	Thr 850	Ile	Val	Asp	Val	Leu Val 930	glu
Pro	Val	Asn 865	Сув	Thr	Cyв	Thr	Cys (
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lle Met Val Arg Lys 975	Pro	Сув	Glu	Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu 1030 1040	Pro Asp 1055	Çya	Ala
Arg 975	Pro Gly Thr Cys 990	Cys Leu Ala 1005	Asn	Met	Pro 1	Ser	Ser
Val	Thr 990	Leu	Val	Asn	Thr	Ala Ser 1070	Сув
Met	$_{ m G1y}$		Авр	Ile	Val	Arg	Thr (
	Pro	Thr	Val ; 1020	Cya	Glu	Ser Arg	Phe
Gly Tyr 970	нів	Tyr	Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val 1010 1020	Arg (Glu Pro Gly Tyr Glu Val Thr 1050	Ala	Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys 1075
G1y 970	Aen Glu Cye Arg 985	Pro Gly Ser 1000	Ser	Gly	Gly '	Сув	31у (
Cys Asp Arg	Сув 985	Gly	Gly	His	Pro	Glu (1065	31u (
Авр	Glu	Pro (Ser	Thr	Glu	Авр (Thr (
2	Asn	Ser	Gln (1015	Cya	Сув	Val	Asn
Сув Glu 965	Ile	Asn	Бly	Ile (Cys Ser Cys 1045	Asp	ren '
	Asp	Val	Val	Gly	Сув ; 1045	Arg	7,4B
Ser Tyr His	Cys Gln Asp 980	Cys Val Asn Ser	1 7	Pro		Cys Arg Asp Val Asp Glu Cys Ala 1060	ner Cen
Tyr	Сув	Arg 995	Gly	Thr	Phe Arg	Gly (Gly 1 1075
Ser		Gly	Glu (1010	neg	Ser	Гув (Thr (
Gly	Gly His	Asp Gly Arg 995	Glu	Cys Leu Thr 1025	Gly :	Гув 1	Pro 1
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Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro

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Cys Gin Ser Giy Tyr Trp Val Asn Glu Asp Giy Thr Ala Cys Glu Asp 1090 1100	Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr 1105 1110	Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg 1125 1135	Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly 1140	Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser 1155	Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met 1170	Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu Hís Cys Ala Pro Hís 1185 1180
cys Gin Ser Gi	Leu Asp Glu Cy 1105	Asn Thr Val Gly Ser Phe Ser 1125	Pro Asn Pro Leu (Pro Gln Ser Ser 1155	Tyr Gln Cys Lev 1170	Cys Glu Asp Val 1185
	ហ		10	Ļ	1	20

Gly Thr Arg Cys Gln Asp Val Asp 1225 1230	FIO GLY GLY HIB CYB VAL ABN Thr Glu 1240	Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro 1255	Сув Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp 1270	Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg 1285	Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp 1300	Cys Ala Asn Asp Thr Val Cys Gly Asn His 1320	Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln 1330
Gly Phe Ala Ser Ala Glu C 1220	Cys Aid Aid inr Asp Fro Cys 5 1235	Gly Ser Phe Ser Cys Leu Cys (1250	Asp Ser Gly Glu 1265	Pro Val	Cys Ile Leu Asp Cys Gln P 1300	Cys Ile Asp Ile Asp Glu 1315	Gly Phe Cys Asp Asn Thr Asp (1330
	U)		10	15		70	

	Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu 1345	n Glu 1360
ហ	Сув Glu Leu Met Ala Val Сув Gly Aвр Ala Leu Сув Glu 1365	Aen Val 1375
	Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp 1380 1380	т Авр
10	10 Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile 1395	g Ile
ū	Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met 1410 1415	t Glu
3	Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile 1425 1435	Leu Gly 1440
20	Gln Aen Ser Thr Gln Ala Glu Cys Cys Thr Gln Gly Ala 1445	Arg Trp 1455
	Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe	e Ser

ŢŢ.	Phe	Gly 1520	Arg	Gly	Pro	Ser	Lys 1600
Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp 1475	Leu	Pro Gly 152	Ser Arg 1535	Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly 1540 1545	Cys Val Asn Glu Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro 1555 1565	Ser	Trp Lys 160
Gly 5	Val	Val	Ser	Glu 1 1550	Asn	Thr	Сув
Glu (1485	Tyr Thr Asp Ala Asp Glu Cys Val 1495	Ser Asn Ile 1515	Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala 1525	Сув	Сув 1 1565	Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr 1570 1576	Pro Asp His Asp Ile His Met Asp Ile Cys 1590
val	Glu (. Asn 5	ABP	Ala	Leu	Asn 1 1580	Авр
Pro	Asp		Tyr 0	Leu	Ç	Val	Met)
: Ile	Ala	Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys 1505	His 1530	. Asp 5	His	Cys	His
7. Tyx	: Авр	Arg	<u> </u>	Gln	. Phe	Arg	Ile
1480	: Thz)5	1 G1y	, Gly	ζ ζ	. Ser] 1560	, Gln 'S	Авр
, Glr	1495	ABE	1 Pro	ı Glu	, G1y	: Gly (1575	His 0
r ਕੀ	Phe Gly Gln Thr Met 1490	8 Gln 2 1510	8 A81 25	s Asr	n Glu	ı Ser	1590
Se	n Th	n Cy	u Cys	p H11	n Glr	o Let	e Pro
Сув Рr 1475	۲ <u>G</u>	r Le	a Le	n Asp 1540	1 Ası 55	ı Ası	Thr Glu Asp Phe 1585
и Су 14	Phe Gl	o Al	C.	8 G];	s Val 2 1555	r Lei 70	1 Asj
n Le		y Pr. 05	r IJ	ζ̈ν	Š	1 Thr 1	r G1. 35
ម	Thr	Gly]	Tyr	Ţ.	Glu	Leu	Thr (
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Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr 1605	Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln 1620	Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys 1635 1640	Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg 1650	Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu 1665 1670 1670	Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp 1685 1690	Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp 1700 1700	Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro 1715
	S		10	r u	3	20	

Leu	
Gly	
Glu	
Phe	_
Ser	174(
Ala	
Pro	
Pro	
Glu	••
Ser	1735
His	
Ser	
Ala	
Leu	
Tyr	1730
His	

Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys

Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln

Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp 1790 1785 1780

Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr 1795

Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro 1815 1810

Gly Pro Pro His Cys Ala Ala Lys Glu *

1830

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CLAIMS

 A method for transferring a nucleic acid segment into bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated nucleic acid segment so as to transfer said nucleic acid segment into said cells.

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- 2. The method of claim 1, wherein said cells are located within a bone progenitor tissue site of an animal and said tissue site is contacted with said composition so as to promote nucleic acid transfer into bone progenitor cells in situ.
- 3. The method of claim 2, wherein the contacting process comprises bringing said isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment composition and bringing said matrix-nucleic acid segment composition into contact with said tissue site.

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- 4. The use of a composition comprising an isolated nucleic acid segment and a bone-compatible matrix in the preparation of a formulation or medicament for transferring a nucleic acid segment into bone progenitor cells.
- 5. A use according to claim 4, wherein said formulation or medicament is intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

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6. A use according to claim 5, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment formulation or medicament intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

7. A use according to claim 6, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-nucleic acid segment formulation or medicament.

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8. A use according to claim 6, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.

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9. A use according to claim 8, wherein said formulation or medicament further comprises a radiographic agent.

- 10. A use according to claim 8, wherein said formulation or medicament further comprises a paramagnetic ion.
- 30 11. A use according to claim 8, wherein said formulation or medicament further comprises a radioactive ion.
- 12. A use according to claim 4, wherein said nucleic35 acid segment is a DNA molecule.

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13. A use according to claim 4, wherein said nucleic acid segment is an RNA molecule.

- 5 14. A use according to claim 4, wherein said nucleic acid segment is an antisense nucleic acid molecule.
- 15. A use according to claim 4, wherein said nucleic

 10 acid segment is a linear nucleic acid molecule, a
 plasmid, a recombinant insert within the genome of a
 recombinant virus, or a nucleic acid segment associated
 with a liposome.

16. A use according to claim 15, wherein said nucleic acid segment is a nucleic acid segment associated with a liposome.

17. A use according to claim 4, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

18. A use according to claim 6, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

19. A use according to claim 18, wherein said bone-compatible matrix is a titanium matrix.

35

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20. A use according to claim 19, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

- 21. A use according to claim 18, wherein said bone-compatible matrix is a collagen preparation.
- 22. A use according to claim 21, wherein said bonecompatible matrix is a type II collagen preparation.
- 23. A use according to claim 22, wherein said bonecompatible matrix is a type II collagen preparation obtained from hyaline cartilage.
- 24. A use according to claim 22, wherein said bone-compatible matrix is a recombinant type II collagen preparation.
- 25. A use according to claim 22, wherein said bonecompatible matrix is a mineralized type II collagen preparation.
- 26. A method of stimulating bone progenitor cells,
 30 comprising contacting bone progenitor cells with a composition comprising an isolated osteotropic gene so as to promote expression of said gene in said cells.
- 35 27. The method of claim 26, wherein said cells are located within a bone progenitor tissue site of an animal

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and said tissue site is contacted with said composition so as to promote bone tissue growth.

28. The method of claim 27, wherein the contacting process comprises bringing said osteotropic gene into contact with a bone-compatible matrix to form a matrix-gene composition and bringing said matrix-gene composition into contact with said tissue site.

10

- 29. The use of a composition comprising an isolated osteotropic gene in the preparation of a formulation or medicament for use in promoting expression of the gene in bone progenitor cells and for stimulating said bone progenitor cells.
- 30. A use according to claim 29, wherein said
 formulation or medicament is intended for use in
 promoting expression of the gene in bone progenitor cells
 within a bone progenitor tissue site of an animal and for
 stimulating said bone progenitor cells to promote bone
 tissue growth.

25

31. A use according to claim 30, wherein said formulation or medicament is prepared by bringing an osteotropic gene into contact with a bone-compatible matrix to form a matrix-gene formulation or medicament intended for use in promoting expression of the gene in bone progenitor cells within a bone progenitor tissue site of an animal and for stimulating said bone progenitor cells to promote bone tissue growth.

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32. A use according to claim 31, wherein said formulation or medicament is prepared by bringing an osteotropic gene into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrixgene formulation or medicament.

- 33. A use according to claim 31, wherein said formulation or medicament further comprises a detectable
 10 agent for use in an imaging modality.
- 34. A use according to claim 33, wherein said formulation or medicament further comprises a radiographic agent.
- 35. A use according to claim 34, wherein said formulation or medicament further comprises calcium phosphate.
 - 36. A use according to claim 33, wherein said formulation or medicament further comprises a paramagnetic ion.

25

37. A use according to claim 36, wherein said formulation or medicament further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

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38. A use according to claim 33, wherein said formulation or medicament further comprises a radioactive ion.

5

39. A use according to claim 38, wherein said formulation or medicament further comprises iodine¹³¹, iodine¹²³, technicium^{99m}, indium¹¹¹, rhenium¹⁸⁶, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.

10

40. A use according to claim 29, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adenoassociated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated with a liposome.

20

41. A use according to claim 40, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

25

- 42. A use according to claim 29, wherein said osteotropic gene is a parathyroid hormone (PTH) gene, a bone morphogenetic protein (BMP) gene, a growth factor gene, a growth factor gene, a cytokine gene or a chemotactic factor gene.
- 43. A use according to claim 42, wherein said osteotropic gene is a transforming growth factor (TGF)

 35 gene, a fibroblast growth factor (FGF) gene, a granulocyte/macrophage colony stimulating factor (GMCSF)

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gene, an epidermal growth factor (EGF) gene, a platelet derived growth factor (PDGF) gene, an insulin-like growth factor (IGF) gene, or a leukemia inhibitory factor (LIF) gene.

5

- 44. A use according to claim 43, wherein said osteotropic gene is a TGF- α , TGF- β 1 or TGF- β 2 gene.
- 10

- 45. A use according to claim 42, wherein said osteotropic gene is a PTH gene.
- 15 46. A use according to claim 42, wherein said osteotropic gene is a BMP gene.
- 47. A use according to claim 46, wherein said osteotropic gene is a BMP-2 or BMP-4 gene.
 - 48. A use according to claim 31, wherein said bone-compatible matrix is a collagenous, metal,
- 25 hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.
- 49. A use according to claim 48, wherein said bone-30 compatible matrix is a titanium matrix.
 - 50. A use according to claim 49, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

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- 51. A use according to claim 48, wherein said bone-compatible matrix is a collagen preparation.
- 5 52. A use according to claim 51, wherein said bone-compatible matrix is a type II collagen preparation.
- 53. A use according to claim 52, wherein said bone-10 compatible matrix is a type II collagen preparation obtained from hyaline cartilage.
- 54. A use according to claim 52, wherein said bone-15 compatible matrix is a recombinant type II collagen preparation.
- 55. A use according to claim 52, wherein said bone-20 compatible matrix is a mineralized type II collagen preparation.
- 56. A use according to claim 31, wherein said matrixgene composition is applied to a bone fracture site in said animal.
- 57. A use according to claim 31, wherein said matrix-30 gene composition is implanted within a bone cavity site in said animal.
- 58. A use according to claim 31, wherein said bone cavity site is the result of dental or periodontal surgery or the removal of an osteosarcoma.

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- 59. A composition comprising an isolated nucleic acid segment in association with a bone-compatible matrix.
- 5 60. The composition of claim 59, wherein said nucleic acid segment is a DNA molecule.
- 61. The composition of claim 59, wherein said nucleic acid segment is an RNA molecule.
 - 62. The composition of claim 59, wherein said nucleic acid segment is an antisense nucleic acid molecule.

63. The composition of claim 59, wherein said nucleic acid segment is a linear nucleic acid molecule, a

- plasmid, a recombinant insert within the genome of a 20 recombinant virus, or a nucleic acid segment associated with a liposome.
 - 64. The composition of claim 63, wherein said nucleic acid segment is associated with a liposome.
- 65. The composition of claim 59, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

25

66. The composition of claim 59, wherein said bonecompatible matrix is a collagenous, titanium,
hydroxylapatite, hydroxylapatite-coated titanium,
bioglass, aluminate, bioceramic, acrylic ester polymer or

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lactic acid polymer matrix.

67. The composition of claim 66, wherein said bone-5 compatible matrix is a collagen preparation.

68. The composition of claim 67, wherein said bone-compatible matrix is a type II collagen preparation.

10

69. The composition of claim 68, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

15

70. The composition of claim 68, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

20

71. The composition of claim 68, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

25

72. The composition of claim 59, further defined as a syringeable composition.

30

73. The composition of claim 59, wherein said composition further comprises a detectable agent for use in an imaging modality.

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- 74. The composition of claim 73, wherein said composition further comprises a radiographic agent.
- 5 75. The composition of claim 73, wherein said composition further comprises a paramagnetic ion.
- 76. The composition of claim 73, wherein said10 composition further comprises a radioactive ion.
- 77. A composition comprising an isolated osteotropic gene in association with a bone-compatible matrix, said composition being capable of stimulating bone growth when administered to a bone progenitor tissue site of an animal.
- 78. The composition of claim 77, wherein said

 20 osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adenoassociated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated

 25 with a liposome.
- 79. The composition of claim 78, wherein said osteotropic gene is in the form of an osteotropic gene 30 associated with a liposome.
- 80. The composition of claim 77, wherein said osteotropic gene is a PTH, BMP, TGF-α, TGF-β1, TGF-β2,
 FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

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81. The composition claim 80, wherein said osteotropic gene is a TGF- α , TGF- β 1, TGF- β 2, PTH, BMP-2 or BMP-4 gene.

5

82. The composition of claim 77, wherein said bone-compatible matrix is a collagenous, metal, hydroxyapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

10

83. The composition of claim 82, wherein said bone-compatible matrix is a titanium matrix.

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84. The composition of claim 83, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

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- 85. The composition of claim 82, wherein said bone-compatible matrix is a collagen preparation.
- 25 86. The composition of claim 85, wherein said bone-compatible matrix is a type II collagen preparation.
- 87. The composition of claim 86, wherein said bone-30 compatible matrix is a type II collagen preparation obtained from hyaline cartilage.
 - 88. The composition of claim 86, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

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89. The composition of claim 86, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

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90. The composition of claim 77, further defined as comprising an isolated osteotropic gene in association with a bone-compatible matrix and a pluronic agent, the composition forming a syringeable composition.

10

91. The composition of claim 77, wherein said composition further comprises a detectable agent for use in an imaging modality.

15

92. The composition of claim 91, wherein said composition further comprises a radiographic agent.

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- 93. The composition of claim 92, wherein said composition further comprises calcium phosphate.
- 25 94. The composition of claim 91, wherein said composition further comprises a paramagnetic ion.
- 95. The composition of claim 94, wherein said

 composition further comprises chromium (III), manganese
 (II), iron (III), iron (II), cobalt (II), nickel (II),
 copper (II), neodymium (III), samarium (III), ytterbium
 (III), gadolinium (III), vanadium (II), terbium (III),
 dysprosium (III), holmium (III) or erbium (III).

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- 96. The composition of claim 91, wherein said composition further comprises a radioactive ion.
- 5 97. The composition of claim 96, wherein said composition further comprises iodine¹³¹, iodine¹²³, technicium^{95m}, indium¹¹¹, rhenium¹⁸⁶, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.
- 98. A kit comprising, in suitable container means, a pharmaceutically acceptable bone-compatible matrix and a pharmaceutically acceptable osteotropic gene preparation.
- 99. The kit of claim 98, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer 20 matrix.

15

.25

- 100. The kit of claim 99, wherein said bone-compatible matrix is a titanium matrix.
- 101. The kit of claim 99, wherein said bone-compatible matrix is a hydroxylapatite-coated titanium matrix.
- 102. The kit of claim 99, wherein said bone-compatible matrix is a collagenous matrix.
- 103. The kit of claim 102, wherein said bone-compatible matrix is a type II collagen matrix.

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104. The kit of claim 103, wherein said bone-compatible matrix is a type II collagen matrix obtained from hyaline cartilage.

- 5
 105. The kit of claim 103, wherein said bone-compatible matrix is a recombinant type II collagen matrix.
- 10 106. The kit of claim 103, wherein said bone-compatible matrix is a mineralized type II collagen matrix.
- 107. The kit of claim 98, wherein said osteotropic gene preparation comprises a linear osteotropic gene, a plasmid including an osteotropic gene, a recombinant virus having a genome that includes an osteotropic gene or an osteotropic gene associated with a liposome.
- 108. The kit of claim 98, wherein said osteotropic gene preparation comprises a lyophilized gene preparation.
- 25 109. The kit of claim 98, wherein said osteotropic gene preparation comprises a PTH, TGF, BMP, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.
- 30 110. The kit of claim 109, wherein said osteotropic gene preparation comprises a PTH, TGF-&1, TGF-&2, TGF-&3, BMP-2 or a BMP-4 gene.
- 35 111. The kit of claim 98, further comprising a pluronic agent.

- 112. The kit of claim 98, further comprising a detectable agent for use in an imaging modality.
- 113. The kit of claim 112, wherein said composition further comprises a radiographic agent.
- 10 114. The kit of claim 113, wherein said composition further comprises calcium phosphate.
- 115. The kit of claim 112, wherein said composition further comprises a paramagnetic ion.
- 116. The kit of claim 115, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (III), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).
- 25
 117. The kit of claim 112, wherein said composition further comprises a radioactive ion.
- 118. The kit of claim 117, wherein said composition further comprises iodine¹³¹, iodine¹²³, technicium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.
- 119. The kit of claim 98, wherein said bone-compatible

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matrix and said osteotropic gene preparation are present within a single container means.

- 5 120. The kit of claim 119, wherein said container means is a syringe or pipette.
- 121. The kit of claim 98, wherein said bone-compatible
 matrix and said osteotropic gene preparation are present
 within distinct container means.
- 122. The kit of claim 98, further comprising a third container means comprising a pharmaceutically acceptable diluent.
 - 123. The kit of claim 98, further comprising a syringe, pipette or forceps.

20

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124. An osteotropic device, comprising an isolated osteotropic gene capable of expression in bone progenitor cells, the gene associated with an amount of a bone-compatible matrix effective to absorb said gene, wherein said device is capable of stimulating bone formation when implanted within a bone progenitor tissue site of an animal.

- 125. The device of claim 124, wherein said device is a titanium or a hydroxylapatite-coated titanium device.
- 35 126. The device of claim 124, wherein said device is shaped to join a bone fracture site in said animal.

127. The device of claim 124, wherein said device is shaped to fill a bone cavity site in said animal.

5

128. The device of claim 124, wherein said device is an artificial joint.

10 129. A DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous

sequence from SEQ ID NO:3.

15

130. The DNA segment of claim 129, comprising an isolated gene that includes a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2.

20

131. A composition comprising a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3.

25

132. A method for stimulating a bone progenitor cell, comprising contacting a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen.

30

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133. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for stimulating a bone progenitor cell.

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134. A use according to claim 133, wherein said composition comprises type II collagen purified from hyaline cartilage.

5 135. A use according to claim 133, wherein said composition comprises recombinant type II collagen.

- 10 136. A use according to claim 133, wherein said composition comprises type II collagen further supplemented with minerals.
- 15 137. A use according to claim 136, wherein said composition comprises type II collagen further supplemented with calcium.
- 20 138. A use according to claim 133, wherein said composition comprises between about 1 mg and about 500 mg of type II collagen.
- 25 139. A use according to claim 138, wherein said composition comprises between about 1 mg and about 100 mg of type II collagen.
- 30 140. A use according to claim 139, wherein said . composition comprises about 10 mg of type II collagen.
- 141. A use according to claim 133, wherein said

 composition comprises type II collagen in combination with a nucleic acid segment that encodes a polypeptide or

- 268 -

protein that stimulates bone progenitor cells when expressed in said cells.

- 5 142. A use according to claim 141, wherein said nucleic acid segment comprises an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or chemotactic factor gene.
- 143. A use according to claim 142, wherein said nucleic acid segment comprises an isolated BMP gene.
- 15 144. A use according to claim 143, wherein said nucleic acid segment comprises an isolated BMP-2 or BMP-4 gene.
- 145. A use according to claim 141, wherein said20 composition further comprises a detectable agent for use in an imaging modality.
- 146. A use according to claim 133, wherein said
 25 formulation or medicament is intended for use in
 stimulating a bone progenitor cell located within a bone
 progenitor tissue site of an animal and for promoting
 bone tissue growth.
- 147. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone cavity site in an animal and for promoting bone tissue growth in said bone cavity site.

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148. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone fracture site in an animal and for promoting bone tissue growth in said bone fracture site

149. A method for promoting bone growth, comprising contacting a bone progenitor tissue site of an animal with a composition comprising type II collagen in an amount effective to activate bone progenitor cells of said tissue site.

150. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for promoting bone growth in a bone progenitor tissue site of an animal.

20

- 151. A use according to claim 150, wherein said composition comprises recombinant type II collagen.
- 25 152. A use according to claim 150, wherein said composition comprises type II collagen further supplemented with minerals.
- 30 153. A use according to claim 150, wherein said composition comprises type II collagen and an osteotropic gene in a combined amount effective to activate bone progenitor cells of said tissue site.

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154. A use according to claim 153, wherein said composition comprises type II collagen in combination with a PTH, TGF-S or BMP gene.

5

155. A use according to claim 153, wherein said composition further comprises a detectable agent for use in an imaging modality.

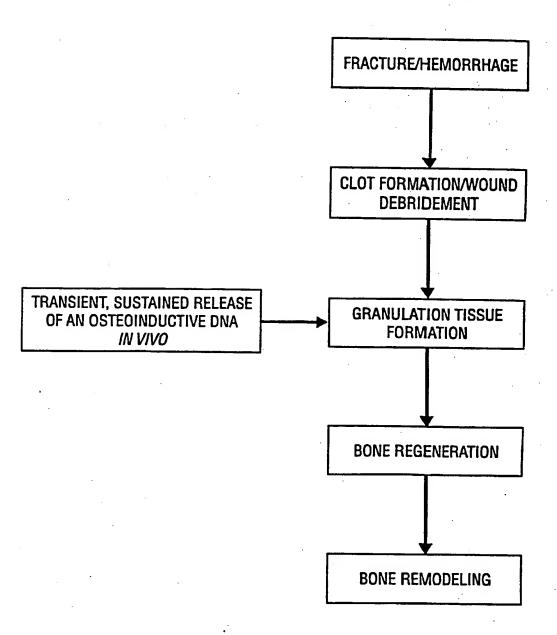
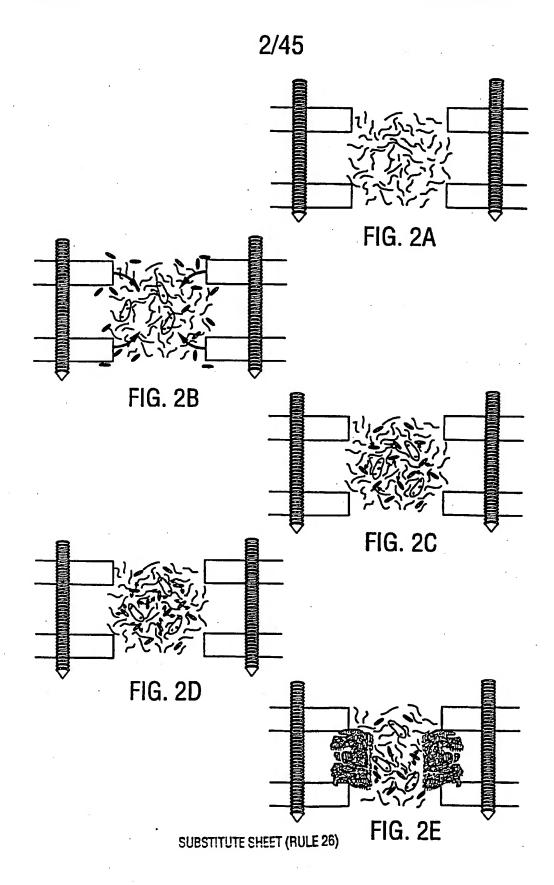


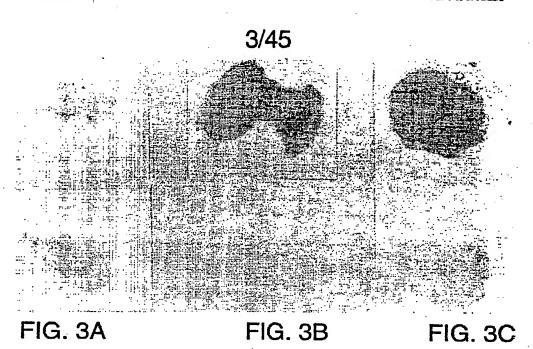
FIG. 1

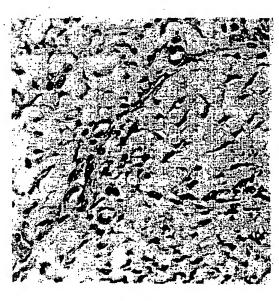
SUBSTITUTE SHEET (RULE 26)





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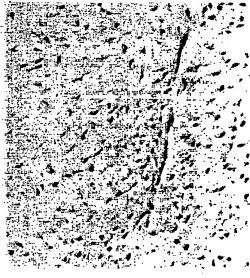


FIG.3D

FIG. 3E

SUBSTITUTE SHEET (RULE 26)

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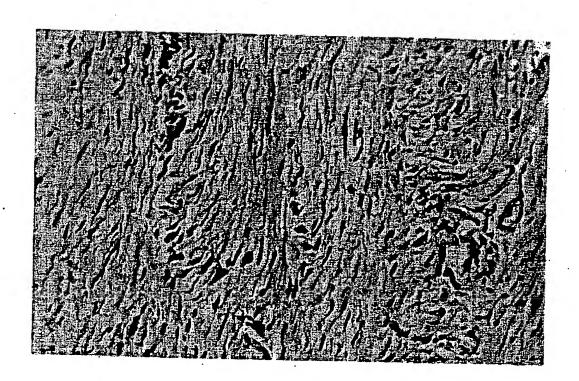
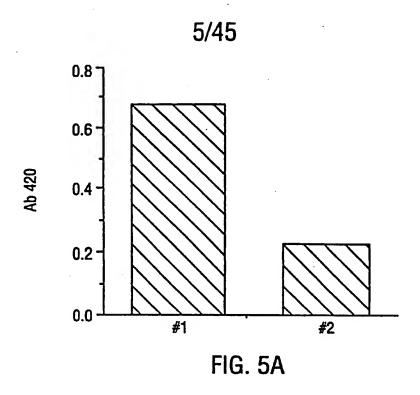


FIG. 4
SUBSTITUTE SHEET (RULE 26)



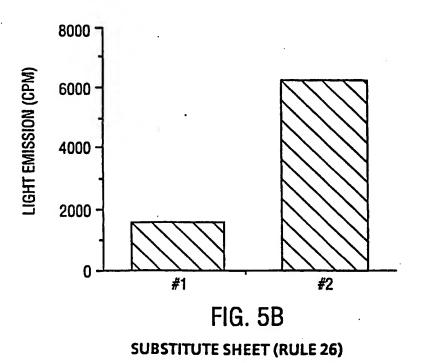




FIG. 6A



FIG. 6B



FIG. 6C



FIG. 6D

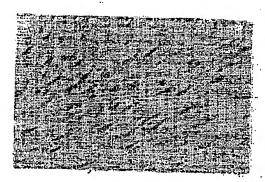


FIG. 7A

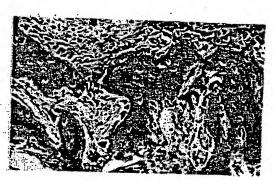


FIG. 7B



FIG. 8A

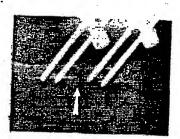


FIG. 8B



FIG. 8C

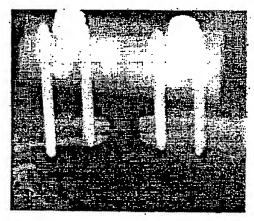


FIG. 9A

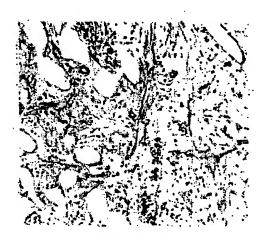
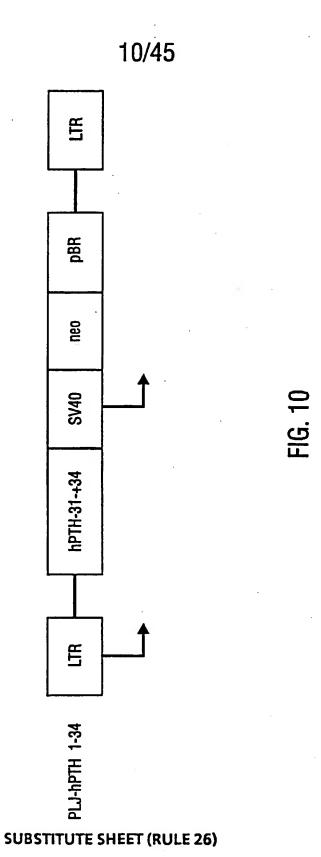


FIG. 9B



1 2 3 4

4.3-

FIG. 11

4.4 - hPTH1-34

.5 – β-gal

Neo Neo

β-actin

FIG. 12

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CONTROL FEMUR

OSTEOTOMY FEMUR

FIG. 13

SUBSTITUTE SHEET (RULE 26)



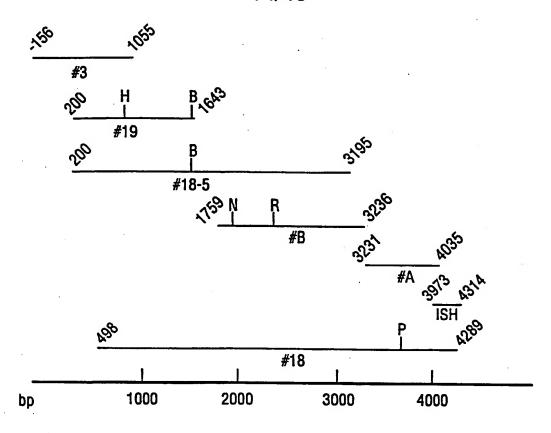
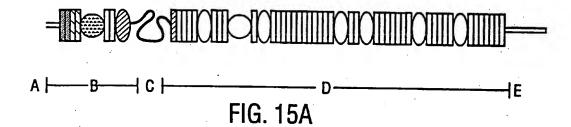


FIG. 14



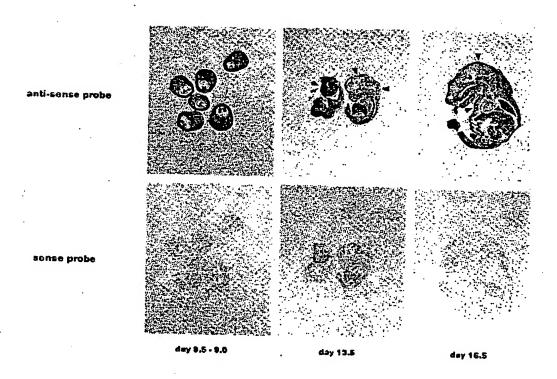


FIG. 16



FIG. 17A

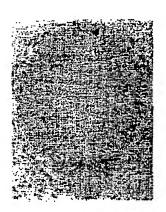


FIG. 17B

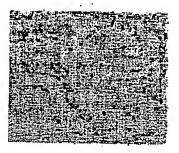


FIG. 17C

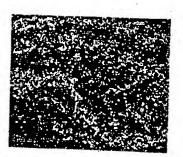


FIG. 17D

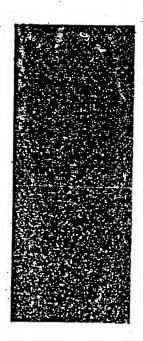


FIG. 18A

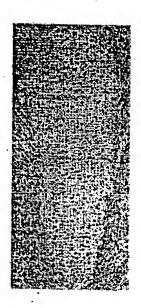


FIG. 18B

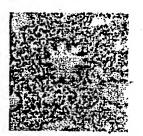


FIG. 18C



FIG. 18D

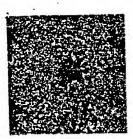


FIG. 18E

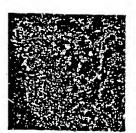


FIG. 18F



FIG. 18G



FIG. 18H



FIG. 181



FIG. 18J



FIG. 18K



FIG. 18L



FIG. 18M



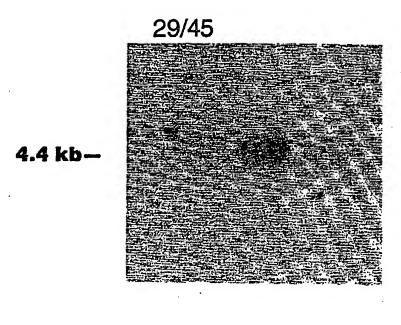
FIG. 18N



FIG. 180



FIG. 18P



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5 14 28 FIG. 19



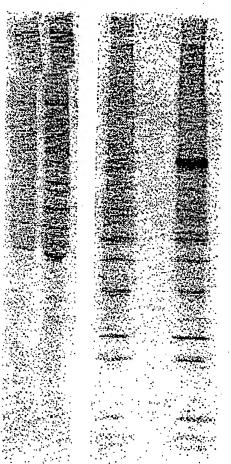
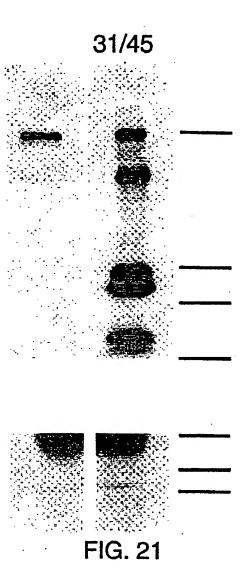


FIG. 20



RECTIFIED SHEET (RULE 91)
ISA/EP

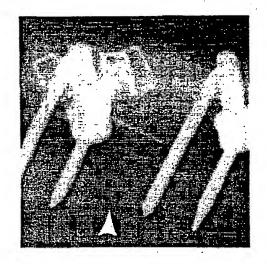


FIG. 22A

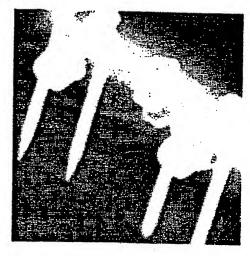


FIG. 22B



FIG. 22C

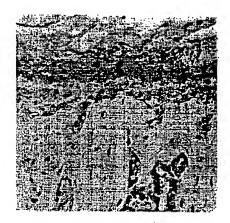


FIG. 23A

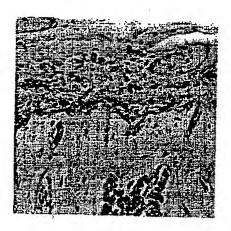


FIG. 23B

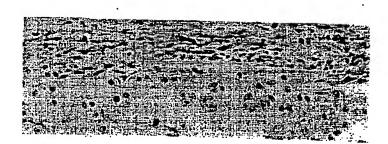


FIG. 23C

HIPGNRHIMV VLICQVLLGG ATDABLMPET GKKKVAEIQG HAGGRRSGQS HELLRDFEAT LLQMFGLRRR popskbavip dymsdlyrlo sceeeeebgs ogtgleyper passantvbs phheehleni pgtsbbafr QGBGNWAQLR PLLVTFGHDG RGHTLTRRSA KRSPKHHPQR SSKKNKNCRR HSLYYDFSDV GWNDWIVAPP GYQAFYCHGD CPFPLADHLN ppenlbbipe nevisbaelr lpreqvdqgp dweqgfhrwn iyevhrppae mypghlitrl ldtblurhny BINHAIVQIL VNSVNBBIPK ACCVPIELSA IBMLYLDEYD KVVLKNYQEM VVEGCGCRYP YDVPDYA **QHVSISRSLP** TRHETEDVSP AVLRWTREKQ PNYGLAIEVT HLHQTRTHQG

FIG. 24

ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG CTG GGC 54 M R Q A A L G L L A L L L L A L L G 18 CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG GCG GGG GCG GGG 108 G CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT GTG ATC TGC AAG CGG ACC 162 TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT CAG CAG GGC TCC AAC ATG ACG CTC 216 C Q Q G S N M T L 72 Q C R D S ATC GGA GAG AAC GGC CAC AGC ACC GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG 270 G H G S GTG GTG TGC CCT CTA CCC TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG 324 V V C P L P C M N G G Q C S S R N O 108 TGC CTG TGT CCC CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA 378 R F С ACC GGA GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432 R A ACA GGC CCG CTG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC AAA CAC 486 GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG GGG GAG GGT CCT 540 IAD P G CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG CCA GGA CAA ATC TCG GCA 594 GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC GTG CGT GTC CAT CAC CCT CCT GAA 648 GCT TCC GTT CAG GTG CAC CGC ATC GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT 702 N G TCC CAG CAC TTG CTG CCG CAT CCC AAG CCC CAG CAC CCG AGG CCA CCC ACT CAA 756 AAG CCA CTG GGC CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC 810 F D AAC CCT TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT 864 N P L P G L T K Q E D C C G 8 GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA GGG GTG 918 KCHKCPQLQ CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC CCC CAG GGC TAC 972 ANG AGG CTC ANC AGC ACC CAC TGC CAG GAT ATC ANC GAN TGT GCG ATG CCC GGG 1026 K R L N S T H C Q D I N E C A M P G 342

FIG. 25-1

AAT GTG TGC CAT GGT GAC TGC CTC AAC AAC CCT GGC TCT TAT CGC TGT GTC TGC 1080 C L N N P G S Y R C V CCG CCC GGT CAT AGC TTG GGT CCC CTC GCA GCA CAG TGC ATT GCC GAC AAA CCA 1134 A A O CIA GAG GAG AAG AGC CTG TGT TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC 1188 Ð CCT CTG ACC ACA CGC CTA ACC CGC CAG CTC TGC TGC TGT AGT GTG GGT AAA GCC 1242 TGG GGT GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG 1296 ATC TGC CCC GGC TGG GAA AGG GTA CCA TAT CCT CAC CTC CCA CCA GAC GCT CAC 1350 CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC GGG CCA CCC AAA 1404 G G K R L CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCC CTC GAG GAC ACA GAG 1458 gaa gag aga gga gtg acc atg gat cca cca gtg agt gag gag cga tcg gtg cag 1512 CAG AGC CAC CCC ACT ACC ACC ACC TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC 1566 S P P R TOT CGC CCC TCC CCA CCT ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC 1620 CGA AGT GCA GTG GAG ATC GCC CCC ACT CAG GTC ACA GAG ACC GAT GAG TGC CGA 1674 R .S A V E I A P T Q V T E T D E C R 558 TTG AAC CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC 1728 C G H G Q C v G TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC TGT GTT 1782 GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA GGC ATC TGT ATG 1836 AAC ACT GGT GGC TCC TAC AAT TGT CAC TGC AAC CGA GGC TAC CGC CTC CAC GTG 1890 N GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG AAC GAG TGC GCC AAG CCT CAC CTG 1944 S L N E TGT GGG GAC GGT GGC TTC TGC ATC AAC TTC CCT GGT CAC TAC AAA TGC AAC TGC 1998 TAT CCT GGC TAC CGG CTC AAG GCC TCC CGA CCG CCC ATT TGC GAA GAC ATC GAC 2052 R L ĸ A S R P P I C E D GAG TGT CGC GAC CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC 2106

FIG. 25-2

SUBSTITUTE SHEET (RULE 26)

AGC TTC AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC 2160 R S TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA TGG TGT 2214 E G GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG ATA CGA ACC CGC 2268 R C. T C A Q G I R ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT GAG GCT GGG AAA GTG TGC 2322 D CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC TCT TTC CAG TGT CAG TGC CTC TCC 2376 GGC TAT CAT CTG TCA AGG GAT CGG AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC 2430 S R DRC TTC CCT GCG GCC TGC ATC GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA 2484 S D С I N G TGT CTC TGT CCC CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT 2538 R CKK ATA GAT GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC 2592 E С S Q D P G A CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC CAG GAC 2646 CAG CAT GGG TGT GAG GAG GTG GAG CAG CAC CAC AAG AAG GAG TGC TAC CTT 2700 H H AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG GCT ACC AAT GTC ACT CAG 2754 D CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC TGG GGA GAC CAC TGC GAA ATC TAT 2808 CCC TGT CCA GTC TAC AGC TCA GCC GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA 2862 H S AGG CTA CAC TCA GGA CAA CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC 2916 H S G Q Q H С E L C · ATC GAC GAA TGC ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG 2970 AAC TOG CAG COO GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024 С K AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC TGC AGG 3078 E v E AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC ACT CCG CCG GCA 3132 A 1044 GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG AGC CCG GAG GAG ATG GAG CAC GCC 3186 0 E M

FIG. 25-3

CCA GAG AGA CGT GAA GTG TGC TGG GGC CAG CGA GAG GAC GGC ATG TGT ATG 3240 RRE V WGQR G E GGG CCC CTG GCG GGA CCT GCC CTC ACT TIT GAT GAC TGC TGC TGC CGC CAG CCG 3294 D D CGG CTG GGG TAC CAG TGC AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC 3348 - Q R P G G S CCG ACT TCA CAG AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG 3402 Ş N S W D T GGG AAG TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT 3456 D S S E TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG TGT CCT 3510 R P G G A V GGA GGC TIT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC ATT GAT GAG TGC 3564 D R C V D CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC GAG CGG TGC GTG AAC ACC 3618 G K С <u>T</u> 1206 AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT GGC TTC ACG CGC AGC CGC CCT CAC 3672 FR C V C K A G F R S R GGG CCT GCG TGC CTC AGC GCC GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA 3726 C S A A A I A H GTG ATC GAT CAT CGA GGG TAT TTT CAC TGA

FIG. 25-4

44 99 110 198 220 242 88 154 176 264 308 330 132 352 374 396 Gln Gly Glu Ala Gln Pro LyB Thr Ile Agn Ala Val Pro His Leu Gln Ser Val Ser Сув Gln Ala Ser Aen Arg Pro Gly Gln Ala Ser Gly Ile Ser Pro Thr Asn Leu Leu Gly Val දු Pro Leu Asn Lea Ala Pro Asp Gly Leu Ala Pro Gln Pro Trp Trp Gly Asp Thr Arg G1yAsp Gly Gla Leu Gln Leu Ala Thr Arg Asp His Ala Gly Pro Len Ala Arg Leu Ser Ala Pro Pro His Thr ABP Lys Tyr Gly Leu Lya Pro Len Lea Trp Thr Ser Gly Leu Gly Pro Gln Сyв Gln Tyr His Arg Сyв Asp Сув Len His Leu Lys Val Ser Arg Gln Gly Сув GJ Ser Val Glu Ile Pro Ser Lea Gln Ala Pro Thr His Gly Gln Gly Ser Gly Lys Gln Gln Leu Ser Val Val Gly Gly Gly Leu Leu Pro Lys Lya Ser Ala Leu Ser Thr Val Val Val Thr Pro Pro Asn Len ζλB Ser Gly Leu His Gly Val Ser Ala Lea Pro Pro Leu င်၇ Arg ζλg G1yHis Сув Pro Pro Glu Lea Gly Aen Phe LyB Met Gly LyB Ala Val Gly Asp Pro Phe Сyв Gly Ala Gln Asn Ile Ala Thr Met Сув Ala Agn Glu Gln Pro His Ala Сув Leu Ala His Met Pro Gln Leu Leu Leu Arg Ala Glu Сув Phe Arg Ala Ala Val Ala Leu ÇyB Ala Thr Gly Gln Ala Pro Leu Thr Gly Гув Gly Pro Asp His Arg His Val Asn Pro Pro LyB ζŚ Cya Arg Gly Val Ser Gly Leu Ser Сув Ile Lya Gln Gly Pro Asn Pro Pro Ser Glu Lys Thr Pro glu ABP Val Leu Ile Leu Pro Ser Ala Pro Gly Arg Ser Gln glu Asn Сyв Leu Gly Pro LyB Pro Thr Pro Pro Сyв Phe Gly Ala Glu Pro G1yVal Ile Glu Gly Arg Pro Arg Arg Leu Pro Ala Arg Pro Met Pro Ala Asp Pro Val Ile His Сув Val Trb Asp Pro Val Tyr Thr Gln Asn Pro Ser Gly Pro LyB Val Ser Glu Gla Gln Gly Val Thr Val ABP Len LyB Gly Ser Gly Gly Val Lya Pro Val

FIG. 26-1

506 550 638 572 594 919 999 726 748 682 704 836 828 880 792 902 924 946 Gly Gly Авр Phe Glu Ser Val Val Leu Gla Gly Pro Ser Asn ζŻΒ Leu Leu Asn Ile TYT Thr Ser Gly Val Arg ζŻΒ S S Pro Gly Lea Thr Lea ζŚ CyBVal Arg Asn Agn Ile Pro Asp Tyr Asp Gln Phe Arg Leu Gly Сув Ser Phe Tyr Leu Ser His Arg Gln Met Gly Ile Pro Lya Arg Ser Asp Сув ABP Pro Pro Сув Gly Glu Ser Glu Arg Asn Сув Pro CyB Ser Gly Gly Val Gln S S ABp Glu Gla Gln Tyr Gla Arg Glu His Pro Phe Ala Asp Phe Glu Leu Gln Gln His Pro Val сув Авр гув гув Pro Val Glu Leu His Ser Сув Gly Ile Ser $_{\rm Gly}$ Gly Gly G1yLeu Сув Asp Ser Thr Ser Glu Сув Ser LyB CyB Ser Ala LyB Gly LyB G1yСyв Val Gly lle ζŚ Glu Arg Val Val Val Pro Ile Arg Gly His Lya Gly Glu Сyв His Asn Pro Asp Gly Ser Pro ABP TYT Gla Gly Pro Tyr Pro Leu Ser Asn Tyr Len G1yGlu Сyв Lea Thr Glu Pro Pro. Asp Ser ABP Val Ile Tyr Ala Thr Leu His Ser Pro Gln Arg Pro Arg Ile Glu Pro Gly Gly Arg ζyΒ Ser Asn Tyr Tr Сyв Gly ABP Arg Asn Ala Cys Pro **Tyr** Len Tyr Сyв $_{\rm Gly}$ Thr Glu Len Glu Arg Gly Asp Ser Arg Asn Met Aen Pro Lea Pro His Len Gly Gly TY Pro Thr Cya Ser LyB Сyв Thr Thr G1yVal Val Thr Glu Pro Cys Arg Arg Pro Pro Ser Arg Ile LyB Gln Glu Gly Ser Arg Asn Ser Lya His Ala Gly Val Ser Pro Сув Asn LyB Tyr Pro Pro ζλg Thr Gly Asp Ile cy_B Len gla Asp Asp Çya Gla Glu CyB Thr Leu Ala Сув Gln ABP Pro Arg Agn Asp ζy LyB Arg Glu Arg Thr Asp Ser Сyв His Сув Agn Arg Сув Ile Gln Arg Thr Ser Asp Glu CyB Tyr Сув Ala His Gly Gly ÇyB Leu Gly Gly Сув Val Val Glu ABP Asn Glu Asn LyB Glu Ile Asn Glu Gln His Gln Val Ala Thr Len Gly Gly Ser Thr Val Tyr Leu Tyr Сув ABp Ser Ala LyB Tyr Asp Asp Val LyB reg Zen Val Pro

FIG. 26-2

1012 1034 1056 1078 1100 1122 1144 1166 1188 1210 1232 Gla Lea Glu Arg Ala Ala Ser Ser Ser Asp Glu Cys Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Leu Pro Gln Gly Asp Gly Asn Glu Ser Gly Ile Ser G1yСув Arg Thr ABp Pro Asp Thr Сув Asp Ala Glu Pro Arg Arg Asn Val Val Tyr Сув රු Pro Gly Gln Ser Pro Сув Val Tyr. Asn Сув Gln Ser Cya Gly Ala Val Arg Phe Arg Lyв His Pro Gly Ser ς ζ Ala Glu Arg Phe Asp Asp Pro Pro Сув Ser Arg Gln Gly Cys Ala Thr Gly Leu Gly Asn Tyr Gly Ser Ser Arg Lys Thr Leu Ser LyB gly Ser Lys Ser Ala Сув Сув glu Val Gly Pro Ala Leu Pro Pro Arg Ser Pro Leu Arg Gln Leu Asp Cys Val Leu Tyr Pro Thr Leu Asp Arg Asp Pro ζλ Leu Arg Gln လိုမ Thr ζζg Phe GArg Asp Ile Asp Tyr Pro Ala Pro Asp Gla Gly Arg Gly Asp Ala Thr Cys Arg Phe Trp. Asp Gly Gln Lys Pro Trp Pro Ser Ser Val Pro Asn Сув Leu ABP Trp Gly Ser Asp ζ S Val Ser Glu Asn Glu Trp Thr Arg Сув Glu Arg Val Gly Ser

FIG. 26-3

160 240 320 400 480 560 640 720 800 880 1040 960 1120 1200 1280 1360 1440 1520 1600 1680 1760 ATGGAGAGCA CCTCCCCGCG AGGTCTCCGG TGCCCACAGC TCTGCAGCCA CTCTGGCGCC ATGAGAGCGC CGACCACGG GTCTGGCTTC CAGCCAACTC GGGACAAGTC CCTCTGAGTG CAGCTGCGTC ACCIGICCAG ACTCGGAGAA GCCAICCCCG GGGCCAGCAG CAGAIAGCAG CCCGGGCIGC ACCIITCIGIC GGGGACAGTG TGAGGAGGAA GTGAGGCCAG CAGCCCTGGC GATGTCCAAC CCTGGGGGCT CGGGGACGCT TTCCCGGCAC AGAACCCTGC GCTGGTGAAG AACCGAGGCT ACCCCCTGCT AGCAACAGCA GTGTTACCTG GATTGAAAAT GGCCAGCTGG AGTGTCCCCA AGGATACAAG AGACTGAACC TCAGCCACTG CCAAGATATC AATGAGTGCC TGACCCTGGG CCTCTGCAAG TGGGGACCTT GAACCAGCCG GCCCAGGGGA ACCCGGGATG GCTCGCAGAG GCCGAGGCCA GGAGGCCACC TCGAACCCAG GCGCGCCTGG AAACCCCTCA GCGACCCGCG GCTGCACGGC GAGGGCGGCT CACTGGGAGA AATGTCTGCG TOGACAACAT CAAACAGCAC CAACCACTGT ATCAAACCTG TGTGTCAGCC TCCCTGTCAG GCCCCAGGTC TGCATCTGCC GTTCTGGCTT CCGTGGGGCG CGCTGTGAGG AGGTCATCCC GGCTTGTCGC TGTCTTGATG CACAGAAGCA GCGCCTCAGC CTCTCACCCC CTGTGAGAAG GGTGACACCA CCACCTTGTA CAGTCAGGGT GGCCATGGGC ATGACCCCAA TGCTGGTGTC TGAACCCCTC CCCCTGGGCC GGCACCCCGT ATGGCCAGCT GACCTGTGCC CCGGGTCCGG GGTGAGCTGG TTCTGGGCCA TGTGGCAGTG TTGTCCTGGC CIGCAGCCAA GGIGIACAGI CIGIICCGAG AGCCIGACGC GCCGGICCCC CTCCATCTCG CAGGACTCGA GCTGAGAGAC AGCAGCCCAC AGGCAGCACA TGTGAACCAT GAGAAAATCA AGAAAATCAA AGTCGTCTTC ACCCCCACCA TCTGCAAGCA GCTGCATCGG CCGGGACGAG AGGGAACCTG CAGGGCGAGG CTCTAACCAG CTCGCCTCTG AATCGGTTGT GCCTGTGCCC AGACGCTCAG TGGAGAGGG ACCCGGTCCT GTATCCGGCC ACTGGTGCCA TAGGCCACAG CATTATGGAC AGCACGGTGA ATGGACAGTG TGCTAACCCC CTAGGTAGTC TGACTTCTCA GGAGGACTGC CTGGGGGGTG ACCTCCTGTG CTCCTGCCC ACCCAGACAA GAGGGTCCAG CCTTCCCAGT TTCCTGCCAC CAGGGATGCG CCATCACCAC ACCAGGTGGC CACGGCAACC GCTGTCTAGG AACCAGCTAG TTGGAGGGGC GGTACCACCA CCGTCCAGGA CAGTTCGTCG CCCCTGCCTG AATGGTGGCC TGCCTGTCCC GCAGCCAGAC CGCTGCCTCT GTGCAGATTC GGCCGGAGAG GCCCCTCGGC CACCACCAGT TCATCGCCCC GGATGCATCC AACGGGTGCG GGGAGATACG TACAGCCGCT TCCACCTTCA CCAGAGCCTC TGAGGCCTCT GGATTCCATA GCACTCAGGG TCTGCCAAAT AGAATGCCAG GTGACCAGAA TTCTGCCATC CCTGAAGCAA ATCACCCGCC AGTGTGGAGA GAGTCCAGCC TCGCTGCTCC CCCGCAGCGG GAACCTCACC GTGCCAACAG ATGCCCAACG CTGCCCAGGA CCTGCAGCAG AGGAAGTCTA GCTTTGCCTT GGAGGACAAC TTTGACCCTC CGTATCTATT CACAGGAAAG TGGAAGGTCC GTGCAAATTC TACCCGCTCG CCCTGCAGCA

F1G. 27-1

2640 2480 2800 GACTCGGAGT GCGTGAACAC CAGGGGCAGC TACCTGTGCA CCTGCAGGCC TGGCCTCATG CTGGATCCGT CAAGGAGCCG 2000 2720 3200 3360 3440 3520 3600 3120 3280 GACAAGGCIG ICICCAIGCA GCAGGGACIA IGCIACCGGI CACIGGGGIC IGGIACCIGC ACCCIGCCIT GCACATGTGA ACAGTGTCCC TCCGCCTGTC CCACCTGGGC TGACTCTCGG CACCATCCTT CACAGCCCCC GGAACCCCTG GTCACCCTCG TGGATACAGA CAACACGGAG AATGCCGTCA CTGTGAGGAG GGCTATGTAG CAACATGGAA ACGAGTGTGC AGCGGGTACT CGTCTGCACC TGCTCCTCAC CCCGTGTCCG GGAGGACACT GTGTCAACAC AGAGGGCTCC TTCAGCTGTC GCGAAGATGT TGCCTCTGTC TICTGCCTCT GIGCACCCGG CITTGCTAGT GCTGAGGGG GCACCAGATG CAGACAGCGG AGAATGTTTG GATATTGATG AGTGTGAGGA CCGTGAAGAC CAGGGAGATC TGCCCTGCTG GCCATGGCTA CACCTACTCG AGCTCAGACA CACTGCACCC CTGACAAAGG GGAAGACCAG GCCTCCCAAA CTACACACTA GGCGATGCAG GATATCAACG CTTGGTGACA TGATGACAAC GAGTGTATGA AGCCTGTCAG CCAGCTGGTC AATGGCACCA TGTGTGAGGA CGTGAATGAG TGTGTTGGGG AAGAGCATTG GAAGGTGCAT CGAGATGTGG GCCCCACAGG GGCAACAGAT SGATGAGTGT GAAGGTCCCC AAAGCAGCTG CCGGGGAGGC GAATGCAAGA ACACAGAAGG TTCCTACCAA CAGATATGCT GCTGCAGCCG TGTGGGCAAA GCCTGGGGTA TAGCCCCTTA AGGGAGCAGA CAGAGCAGAG CCAACATCTG TGGCCCTGGG ACCTGTGTGA GAGACCCTCC GGATGCCACT CCAGTGATGT GCTATCCTGG TGCAGTGGTG AATACTGTAG GCTCCTTCTC CTGCAAGGAC TGTGACCAGG GCTACCGGCC CAACCCCTG ACACTGTCAA CTTGTCTGGC TGTACCCATG GAAGGGCTGC TCACCTGCTC CCTGGAGTCT GATTGAGGCT CTACCTGCCC GGGTACCAGG GGCATTCCAG AGAGTCCAGC AGAAGAGCAA GTGATTCCCT CCCAGCCAAG ACTACTGTAC GATATCGATG AGTGTGAGCA GCCCGGGGTG TCAGGAAAGG GGGCTCCTAC TCCTGCCTCT GGCTCCTACA CCCTGGGATA GCCTGTGAAG ACTTGGATGA ATGTGCCTTC CCCCAGACAA CAGCCGAGCC TCGTGCCCCA CGGCCTCTG CCTCAACACG GAGGCCTCCT CCCCCACCTG TACATCATGG GATGTCAATG AGTGTCTGAC CAACTCCCCT GATGCTCCTG TGAGCCGGGC TATGAGGTCA AGGAACTGGC CGGGCAGCCA TGCTCCCCAC GCTGGAGCCT CCAGCTACAC TCAACAGTGT TGATCGGGGC GGAGATGCGT GGGCTCCTTC CAGCCACAGA CAGCCCTCCC GATCACCAAG GCCGAAGAAG TCACAACCAG GGGCGCTGTG CAGAAGCCTT GCAACCACTC TCCATGTTTT GCCCTGGCTA GGAGTGCCAA ACTGCGAGTG TGCCCTGATG GAGCTGTGTA GGGTGAACGA AGATGGCACT TCAACAGCCT GATGAATGTG TGCTTCCTTC CTGCGTATCG AAGCAGAGAG TGGTTCATCG CTGCCTGGCA GCTGTTCAGA GCCTGGACAG CAGACTTTGA TGTGTCTGCA TGAAGGAAGA GAGACACACA GGCTCGTACC CCCTGGTACC GCCAGAGTGG GCTCCTTTA GGCGAGTGCC ACCAGGGCTT CCAGGATGTT TGTGTGAGAC

FIG. 27-2

4560 4720 4880 4160 4240 4320 4400 4640 5040 5200 CCGGTGTGCG GAGCCTGGAG GTGTGAGAAC AGTCCTGGTT CCTACCGCTG CATCCTGGAC TGCCAGCCTG GATTCTATGT 3920 GGAGACTGCA TTGACATAGA TGAATGTGCC AATGACACTG TGTGTGGGAA CCATGGCTTC TGTGACAACA 4000 4480 4800 4960 5120 5280 5360 5440 5502 GCCAGGACTT TGTGAACGAG GCGCCAGTGA GTCCGGACAG AATCCTGGGC CATCTGAGGA GGACAAACCA GCTCAAACAT AGTGCCTGGC CTAGACCTCA CTGCTGGAAA AAGATGGGGA GCTCGGATTG GTGTGGATGT TGAAAACCTC CCAACCCAGC CTAGCCAGCC TGGCCGCTGC TCCTATCGCT AGTGTGTTGA CTGTGCCTTT AATCCCAGAG CCTGCTCTCA CTGGACATIT CACAACGAAT ACATGGACAT TGCTGCTGCC GTGCAACGTG GCGCCCIGCC CCCCCTCACC GAAGACTIGA ACGGGCCTGC ACGACTCTGT GCACACGGTC ACTGTGAGAA CACAGAGGGT GCCTCACTAT GCTGTGAGAA CACTIGCGAC IGCITIGAGG GCITICCAGCI GGAIGCGCCC ACAITIGGCCT ACGATCTGCC CCTCCCTTCT GCCAGGITTAC GTGGCAGAGC CAGGCCCCCC ACACTGTGCG GCCAAGGAGT AG GACCTCACCA TCAGGCTGGG GCGCTCTGTG AGAACGTGGA AGGCTCCTTC CGGGTGGCTG GAGCTCAGAG GGTGGTCCTC TGGAAGGAGC TCTCTGCCAG AATGGCCGAT CCAGCAGGAA GTGCCAGGAT CATGACATCC TCTGCAATCC CTATACAGAA GGAATGTGGC ATCCTGAATG AAAGGCCTGT CCTGGCCTGG TGCCCCTGAG CTGAACTTCA ACGCTCAGCT CCAGATGGGG TACATCCCAG TTCCATTGCC CTTCCCTGAC TGAACACAAT ACCATACCAC TCTGAGGTCT CCGAGGACAC CTGCAGCCCT TGAGTATGGC CTGTGTGACC AGGGCTTCGA TCCAAGCCIT ATCCGCATGG AATGCTACTC ACTCAGGGTG GCACGGAGGA TTGCGTGGGC GCCCAGGAGC TTCGAAGGCC TTCAGGCTGA CTGCCGTCCT TGGTCAAGGT TTGGGCCTGC TATGATGCCT AGAAGGCTCC GGCCAGGCTA TACCTAGGCC TGAGCCTCCT GTGTGGGGAT GTGCTGCTGC TGGCTACCAC GTGTGAACCA TACGACGCAG AAGAAGGACA TCTGCCCCAG TGTGTACTGT ACGACCAGCA CAGCCAGCCC CTCTGTGCCC ATCCACTTCC CTTCTATAAC CACCTGTCCT CTTCCGCTGC TGATGGCAGT TGTGCAACCC CTGTGTGAAC ATGATGTGTG CGGAGCAGGG CCCTGCCTCC GGGAGGCTA CACAGGCCGA TTCAGTCAGC TGCCGATGAA AACGGTGAGT CAGCAATGCG GGAGACAACA ATGGGGCTCC GGCGCCAAAT GGCCTGTGAG CCTTGAGGAG GAACGAGTGT CGGACGGCTC rgrgagcrca AGGACCAGGC CAGAACTCCA CTCAGTTGAA GTATACAGA **IACATITIGCC** GTGGCCAGCG AAAGTCACCA SGCCTGGAGC 'ACGGCCCAG PAGCCAGCCG ACTCAGAACC GTGCGTGTGC GCCACTGTTC AGGCAGAGCG

FIG. 27-3

MESTSPRGLRCPQLCSHSGAMRAPTTARCSGCIQRVRWRGFLPLVLAVLMGTSHAQRDSIGRYEPASRDANRLWHPVGSHPAAAAKVYS	90
LFREPDAPVPGLSPSEWNQPAGGNPGWLAEARARRPPRTQQLRRVQPPVQTRRSHPRGQQQIAARAAPSVARLETPQRPAAARRGRLTGR	180
NVCGGQCCPGWTTSNSTNHCIKPVCQPPCQNRGSCSRPQVCICRSGFRGARCEEVIPEEFDPQNARPVPRRSVERAPGPHRSSEARGSL	270
VTRIQPLVPPSPPPSRRLSQPWPLQQHSGPSRTVRRYPATGANGQLMSNALPSGLELRDSSPQAAHVNHLSPPWGLNLTEKIKKIKVVF	360
TPTICKQTCARGRCANSCEKGDTTTLYSQGGHGHDPKSGFRIYFCQIPCLNGGRCIGRDECWCPANSTGKFCHLPVPQPDREPAGRGSRH	450
RTLLEGPLKQSTFTLPLSNQLASVNPSLVKVQIHHPPEASVQIHQVARVRGELDPVLEDNSVETRASHRPHGNLGHSPWASNSIPARAGE	540
APRPPVLSRHYGLLGQCYLSTVNGQCANPLGSLTSQEDCCGSVGTFWGVTSCAPCPPRQEGPAFPVIENGQLECPQGYKRLNLSHCQDI	630
NECLTLGLCKDSECVNTRGSYLCTCRPGLMLDPSRSRCVSDKAVSMQQGLCYRSLGSGTCTLPLVHRITKQICCCSRVGKAWGSTCEQCP	720
LPGTEAFREICPAGHGYTYSSSDIRLSMRKAEEBELASPLREQTEQSTAPPPGQAERQPLRAATATWIEAETLPDKGDSRAVQITTSAPH	810
LPARVPGDATGRPAPSLPGQGIPESPAEEQVIPSSDVLVTHSPPDFDPCFAGASNICGPGTCVSLPNGYRCVCSPGYQLHPSQDYCTDDN	006
ECMRNPCEGRGRCVNSVGSYSCLCYPGYTLVTLGDTQECQDIDECEQPGVCSGGRCSNTEGSYHCECDRGYIMVRKGHCQDINECRHPGT	990
CPDGRCVNSPGSYTCLACEGYVGQSGSCVDVNECLTPGICTHGRCINMEGSFRCSCEPGYEVTPDKKGCRDVDECASRASCPTGLCLNT	1080
EGSFTCSACQSGYWVNEDGTACEDLDECAFPGVCPTGVCTNTVGSFSCKDCDQGYRPNPLGNRCEDVDECEGPQSSCRGGECKNTEGSYQ	1170
CLCHQGFQLVNGTMCEDVNECVGEEHCAPHGECLNSLGSFFCLCAPGFASAEGGTRCQDVDECAATDPCPGGHCVNTEGSFSCLCETASF	1260
QPSPDSGECLDIDECEDREDPVCGAWRCENSPGSYRCILDCQPGFYVAPNGDCIDIDECANDTVCGNHGFCDNTDGSFRCLCDQGFETSP	1350
SGWECVDVNECELMMAVCGDALCENVEGSFLCLCASDLEEYDAEEGHCRPRVAGAQRIPEVRTEDQAPSLIRMECYSEHNGGPPCSQILG	1440
QNSTQAECCCTQGARWGKACAPCPSEDSVEFSQLCPSGQGYIPVBGAWTFGQTWYTDADBCVLFGPALCQNGRCSNIVPGYICLCNPGYH	1530
YDASSRKCQDHNECQDLACENGECVNQEGSFHCLCNPPLTLDLSGQRCVNTTSSTEDFPDHDIHMDICWKKVTNDVCSQPLRGHHTTYTE	1620
CCCQDGEAWSQQCALCPPRSSEVYAQLCNVARIEAERGAGIHFRPGYEYGPGLDDLPRNLYGPDGAPFYNYLGPEDTAPEPPFSNPASQP	1710
GDNTPVLEPPLQPSELQPHYLASHSEPPASFEGLQABECGILNGCENGRCVRVREGYTCDCFEGFQLDAPTLACVDVNECEDLNGPARLC	1800
AHGHCENTEGSYRCHCSPGYVAEPGPPHCAAKE	1833

FIG. 28

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(74) Agent: PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS

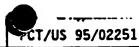
(57) Abstract

Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells in situ and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration in vivo. Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/16 A61K48/00 C07K14/47 A61K38/39
A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K C12N A61L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base committed during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category *	ory * Citation of document, with indication, where appropriate, of the relevant passages Relevant to cla		
Y	WO,A,92 05199 (GENETICS INSTITUTE) 2 April 1992 see page 12 - page 15	17,26, 65, 77-128	
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A	TRENDS IN GENETICS, vol.8, no.3, pages 97 - 102 V. ROSEN ET AL. 'The BMP proteins in bone	1	
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Date of the actual completion of the international search 27 July 1995	Date of mailing of the international search report 29.12.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentiaan 2 NL - 2220 HV Ripsnik Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer SKELLY J.M.

X Further documents are listed in the continuation of box C.

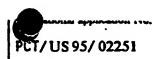
Y Patent family members are listed in annex.



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alegory *	ction) DOCUMENTS CONSIDERED TO BE RELEVANT Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO,A,94 01139 (BAYLOR COLLEGE OF MEDICINE) 20 January 1994 see the whole document	1-16, 18-25, 59-64, 66-76 17,26, 65, 77-128
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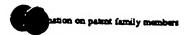


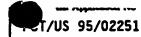
Bex I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This int	ernational search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X	Claims Nos.: 2,3,27,28,149 because they relate to subject matter not required to be searched by this Authority, namely: Remark: Although these claims are directed to a method of treatmnent of (diagnostic method practised on) the human/animal body, the search been carried out and based on the alleged effects of the compound/composition.
2.	Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
з. 🔲	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This Int	ernational Searching Authority found multiple inventions in this international application, as follows:
	1. claims 1-128 2. claims 129-131 3. claims 132-155
	See continuation sheet PCT/ISA/210
ı. 🗌	As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. 🗌	As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
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Remark	The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.





- 1. Compositions devices and methods for therapy of bone diseases comprising nucleic acid
- 2. Latent TGF-beta binding protein-3 (LTBP-3) and DNA encoding it
- 3. Compositions devices and methods for therapy of bone diseases comprising Type II collagen.





Patent document ited in search report	Publication date	Patent family P member(s)		Publication date
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